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Baeyer−Villiger Monooxygenases: Tunable Oxidative Biocatalysts

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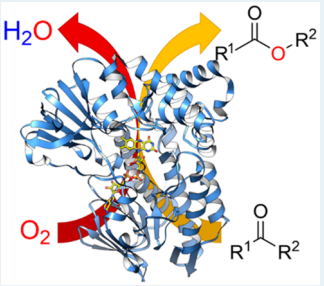
Supporting Information

ABSTRACT: Pollution, accidents, and misinformation have earned the pharmaceutical and chemical industry a poor public reputation, despite their undisputable importance to society. Biotechnological advances hold the promise to enable a future of drastically reduced environmental impact and rigorously more efficient production routes at the same time. This is exemplified in the Baeyer−Villiger reaction, which offers a simple synthetic route to oxidize ketones to esters, but application is hampered by the requirement of hazardous and dangerous reagents. As an attractive alternative, flavin-containing Baeyer−Villiger monooxygenases (BVMOs) have been investigated for their potential as biocatalysts for a long time, and many variants have been characterized. After a general look at the state of biotechnology, we here summarize the literature on biochemical characterizations, mechanistic and structural investigations, as well as enzyme engineering efforts in BVMOs. With a focus on recent developments, we critically outline the advances toward tuning these enzymes suitable for industrial applications.



KEYWORDS: *Baeyer−Villiger, ketone oxidation, peroxyflavin, cyclohexanone monooxygenase, phenylacetone monooxygenase, biocatalysis, protein engineering*

INTRODUCTION

"The field of organic chemistry is exhausted."[1] This notion, which many scientists later judged a fallacy,[2] was not an isolated opinion in the late 19th century[3] from when the quote stems. It is ascribed to chemist Adolf von Baeyer and was supposedly in response to the success in synthesizing glucose,[4] achieved by his earlier student, Emil Fischer. While Fischer was said to share von Baeyer's confidence,[3] their potential rush to judgment did not prevent either of them to later be awarded the Nobel Prize. In the wake of ever more discoveries being made, scientists today largely refrain from such drastically exclusivistic statements and rather call organic chemistry a "mature science".[5]

In hindsight, the time of von Baeyer's controversial statement can in fact be considered as the early days of organic synthesis. Chemistry only started to transform from an analytic to a synthetic discipline after 1828,[6] when Wöhler's urea synthesis was the first proof that organic compounds do not require a "vital force".[7] Similarly to this paradigm shift in chemistry nearly 200 years ago, biology is currently at a turning point.[6,8] Although bread making and beer-brewing can be considered biotechnological processes invented thousands of years ago, the deliberate creation of synthetic biological systems only succeeded in the late 20th century. As much of modern research, biotechnology is an interdisciplinary area,[5] though, a particularly strong overlap with organic synthesis occurs in the field of biocatalysis. One of the main arguments for using enzymes for chemical transformations is that even if inventions in organic chemistry will never exhaust—its major feedstock soon will. Considering the continuing depletion of the world's fossil fuel reserves, a major contemporary challenge represents the switch to synthetic routes starting from alternative building blocks. In the light of the chemical industry and their supplier's historically disastrous impact on the environment,[9] a second challenge is the transition to what has been termed "green chemistry":[10] the choice of building blocks from sustainable sources and the avoidance of hazardous substances. Moreover, with the chemical industry being the single most energy intensive industry sector worldwide,[11] strategies to increase efficiency of chemical processes are urgently needed. Unfortunately, however, such considerations find only reluctant implementation in practice. Despite an increased public pressure due to the poor reputation of the chemical industry,[12] the market economy still nearly irrevocably ensures the design of industrial processes by economical considerations.[13] In research, delaying factors might include the hesitancy to rethink traditional approaches and the fact that environmental considerations are often inconspicuous on lab-scale or out of focus because of the limited scientific prestige.[12,13] In the meantime, biocatalytic transformations have emerged as a profoundly different alternative. Besides the prospect of inherently green catalysts, a hallmark of biocatalysis is product selectivity, as enzymatic reactions arguably allow total control over chemo-, regio-, and enantioselectivity. This renders biocatalysis especially useful for the preparation of pharmaceuticals, where isomeric impurity can have dramatic physiological consequences.[14] One of the biggest assets of enzymes is the prospective of their targeted functional

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evolvability.[15,16] Ever more sophisticated molecular biological methods for DNA manipulation allow easy access to large numbers of enzyme variants, which can be screened for desired activities. Despite being one of the oldest techniques, random mutagenesis libraries continue to be an extremely successful enzyme engineering approach.[15] On the other hand, more rational approaches guided by structural and biochemical data in combination with computational predictions have gained popularity.[17] Although still impractical in most scenarios, the complete de novo design of enzymes has been demonstrated and likely will become a key technology in the future.[18]

Although often seen as a limitation, the usually found restriction of enzymes to aqueous systems and ambient temperatures is also advantageous: these processes not only abide by the principles of green chemistry; the consistency in process conditions also facilitates the design of cascade reactions, which circumvents the need to isolate intermediate products. Cascades can be designed as in vitro processes, in which chemoenzymatic strategies may combine the power of chemo- and biocatalysis.[19] With whole cells as catalysts being the economically most attractive approach,[20] another highly promising procedure is to establish cascades fully in vivo. Recent advances in genetic manipulation techniques greatly accelerated metabolic engineering approaches, allowing the introduction of foreign metabolic pathways into recombinant microbial hosts. These pathways may be of natural origin, partially adapted, or designed entirely de novo. Recent examples of the recombinant production of natural products such as opiods[21,22] or cannabinoids[23] have attracted considerable attention not only in the scientific community. Artificial metabolic routes designed in a " bioretrosynthetic" fashion[24] also allow diverse applications ranging from novel CO_2 fixation strategies[25] to the production of synthetic compounds such as the antimalarial drug artemisinin.[26] With research in this area of biotechnology rapidly developing, it has been suggested to constitute a new field, called synthetic metabolism.[27]

THE BAEYER−VILLIGER REACTION OF PEROXIDES AND MONOOXYGENASES

Presumably, considerations of green chemistry were far from the mind of the before-mentioned Adolf von Baeyer, when 110 years ago, he and his disciple Victor Villiger were experimenting with potassium monopersulfate. In honor of their discovery that this and other peroxides can oxidize ketones to esters, we now call this the Baeyer−Villiger reaction. Although it is a widely known method in organic chemistry nowadays,[28,29] several unsolved difficulties reduce its attractiveness and thus applicability. Especially on large scale, a remaining problem is the shock-sensitivity and explosiveness of many peroxides.[30] Commonly applied peracids are prepared from their corresponding acids using concentrated hydrogen peroxide. As these solutions in high concentrations are prone to ignition and other forms of violent decomposition,[31] they have largely been withdrawn from the market.[32] Reactions with peroxides and peracids furthermore lead to stoichiometric amounts of hazardous waste products. More promise lies in recent achievements of reactions using directly hydrogen peroxide as the oxidant,[33] making use of metal[34] or organocatalysts.[35] However, such processes also require waste treatment, and the catalysts need to be prepared in additional, often complex synthetic routes. In comparison to other oxygenation reactions, examples of asymmetric Baeyer−Villiger oxidations were noted to be scarce and to show limited selectivities, reactivities, and scopes.[33]

Because of these reasons, biocatalysis offers a particularly promising alternative and has attracted considerable attention. So-called Baeyer−Villiger monooxygenases (BVMOs) use the free, abundant, and green oxidant O_2 and only generate water as a byproduct. BVMOs were discovered in the late 1960s by Forney and Markovetz, who were interested in the microbial catabolism of naturally occurring, long-chain methyl ketones. They noticed that the products generated from these compounds by a Pseudomonad were incompatible with terminal methyl oxidation, which was previously assumed to be the only degradation pathway.[36] Subsequently, they were able to identify the responsible enzymatic reaction as a Baeyer−Villiger transformation, dependent on NADPH and molecular oxygen.[37] In parallel, Trudgill and co-workers were investigating microorganisms that are able to grow on non-naturally occurring aliphatics. They identified an oxygen- and NADPH-dependent enzyme from *Acinetobacter calcoaceticus* NCIMB 9871 involved in the microbial metabolism of fossil fuel-derived cyclohexane and suggested that it catalyzes the conversion of cyclohexanone to ϵ-caprolactone.[38] They confirmed their findings by isolating the protein and established that the enzyme contains a flavin adenine dinucleotide cofactor as prosthetic group.[39] This cyclohexanone monooxygenase (AcCHMO) quickly attracted attention because of its broad substrate scope and because caprolactone was already well-known as a precursor to nylon-6.[40,41]

STRUCTURES

Over the decades, AcCHMO has come to be the number one prototype BVMO, despite the failure to obtain its structure. Only recently, in 2019, could a mutant finally be crystallized;[42] however, it remains to be seen whether its structure can serve as a good enough approximation to wild type, considering that it contains 10 active-site substitutions. Fifteen years earlier, the first BVMO crystal structure was solved for phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* (Figure 1A),[43] causing this thermostable enzyme to become a structural prototype. The structure sheds light on a two-residue insertion displayed by PAMO, which was found to be located in the active site and subsequently called "the bulge" (Figure 1B). Eight other BVMOs and various mutant structures followed (Table 1), totaling to 38 structures at the time of writing. Mechanistic insights have mostly been gained by structural studies on CHMO from *Rhodococcus* sp. HI-31 (RhCHMO) and PAMO. Overall, the structures of BVMOs are surprising similar, despite sequence identities of often less than 40%. With the exception of PAMO, many BVMOs are often rather unstable; however, no obvious structural features could be identified as the origin of this stability. A study that compared PAMO's and AcCHMO's tolerance toward cosolvents—a feature frequently shown to be related to thermostability[44]—suggested PAMO's increased number of ionic bridges would cause the lower solvent susceptibility, as it could prevent damage to the secondary and tertiary structure.[45] The same reasoning was given for the higher robustness of a recently crystallized CHMO from *Thermocrispum municipale* (TmCHMO).[46] BVMOs display a multidomain architecture consisting of an FAD-binding, an NADP-binding, and a helical domain. The latter distinguishes BVMOs from other class B flavoprotein monooxygenase families and causes a partial shielding of the active site and the formation of a tunnel toward it. Some BVMO subgroups contain N-terminal extensions of varying length. The structure of such an extension was established in PockeMO, where it forms a long helix and

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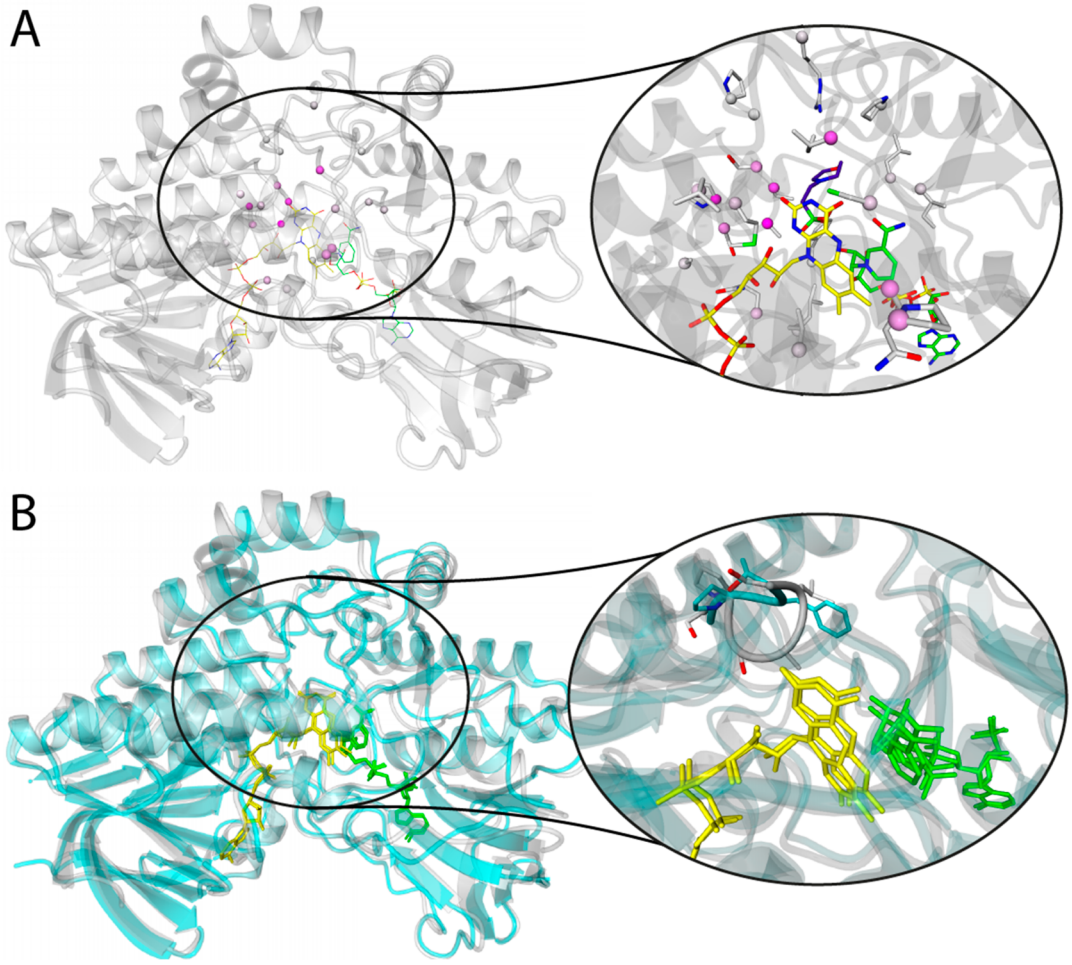

Figure 1. Structures of BVMOs. (A) Crystal structure of PAMO shown as ribbons. FAD, $NADP^+$, and an active-site ligand are shown as sticks with yellow, green, and dark purple carbons, respectively. C-α carbons of residues targeted for engineering are indicated by a sphere. The sphere's color is graded gray to magenta, reflecting the number of reported mutants targeting that site. (B) Superimposition of CHMO and PAMO and close-up view of the bulge, a two-residue insertion displayed by PAMO.

several loops that wrap around the enzyme.[47] This enzyme is more thermostable than most BVMOs, but it is unknown whether the extension plays a role in that. Such a function was suggested for 4-hydroxyacetophenone monooxygenase (HAPMO), where deletion of the extension was not tolerated when exceeding a few amino acids.[48] Removal of only nine amino acids already impaired stability and furthermore decreased the enzyme's tendency to dimerize. Besides FAD, which is found in all BVMO crystal structures, the nicotinamide cofactor is also found in many structures, in accordance with its tight binding to the enzymes.[49] A certain structural mobility of cofactors and loops in BVMOs has been observed, and the debate on its role in catalysis has recently been reviewed.[50] The determination of various BVMO structures has been instrumental for the investigation of their catalytic mechanism.

MECHANISM OF THE BAEYER–VILLIGER REACTION

BVMO catalysis (Scheme 1) is initiated by NADPH binding and subsequent flavin reduction, after which the nicotinamide cofactor adopts a stable position.[52,59] Because the stereochemistry of the transferred hydride is in disagreement with the nicotinamide orientation in the stable position, a potential conformational change of NADPH during the reduction step is currently under discussion.[50] Flavoproteins allow detailed mechanistic studies because of the characteristic absorption spectra traversed by the flavin cofactor during the various states of catalysis (Scheme 2). In BVMOs, a stable peroxyflavin was identified to be the catalytically active species.[62] Formed by the radical reaction[63] of two electron-reduced FAD with molecular oxygen, this spectroscopically observable flavin intermediate was already known from the flavin-dependent aromatic hydroxylases[64] and luciferases.[65] The finding was perhaps rather unsurprising, considering that the chemical Baeyer–Villiger reaction is also afforded by peroxides. However, while with few exceptions,[29] the chemical reaction is acid catalyzed, thus entailing a protonated peroxide, the catalytic flavin species requires a deprotonated peroxy group.[66] While quickly decaying in solution,[67] some BVMOs stabilize this reactive species for several minutes in the absence of a substrate, before its decomposition forms hydrogen peroxide in the "uncoupling" side reaction known to occur in all monooxygenases.[68−71] The exact factors flavoenzymes exert to influence the longevity of both the protonated and unprotonated peroxyflavin are largely unknown, despite reported lifetimes ranging from milliseconds in some oxidases[72] to several minutes or even hours in FMOs[73,74] and luciferases.[75] In BVMOs and other class B monooxygenases, $NADP^+$ was, however, found to be critical for intermediate stabilization, as a manifold increased peroxyflavin

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Table 1. Available BVMO Crystal Structures

name	acronym	source strain	UniProt ID	PDB entries	important residues[a]				ref
					D	R	Ω	bulge	
cyclohexanone monooxygenase	AcCHMO	*Acinetobacter calcoaceticus* NCIMB9871	Q9R2F5	6A37[b]	57	327	W 490	P--F 431−432	42
Aspergillus flavus monooxygenase 838	Af838MO	*Aspergillus flavus* NRRL 3357	B8N653	5J7X	63	337	W 502	PTAF 441−444	51
cyclohexanone monooxygenase	RhCHMO	*Rhodococcus* sp. HI-31	C0STX7	3GWD, 3GWF, 3UCL[c], 4RG3[c], 4RG4[c]	59	329	W 492	P--F 433−434	52−54
cyclohexanone monooxygenase	RpCHMO	*Rhodococcus* sp. Phi1	Q84H73	6ERA[b], 6ER9	60	330	W 493	P--F 434−435	55
cyclohexanone monooxygenase	TmCHMO	*Thermocrispum municipale* DSM 44069	A0A1L1QK39	5M10[c], 5M0Z, 6GQI[c]	59	329	W 492	P--F 433−434	46,56
2-oxo-Δ^3-4,5,5-trimethylcyclo-pentenylacetyl-coenzyme A monooxygenase	OTEMO	*Pseudomonas putida* ATCC 17453	H3JQW0	3UOV, 3UOX, 3UOY, 3UOZ, 3UP4, 3UP5	59	337	W 501	GSTF 440−443	57
phenylacetone monooxygenase	PAMO	*Thermobifida fusca* YX	Q47PU3	1W4X, 2YLR, 2YLS, 2YLT[c], 2YLW[b,c], 2YLX[b,c], 2YLZ[b], 2YM1[b], 2YM2[b], 4C74, 4C77[b], 4D03[b], 4D04[b], 4OVI	66	337	W 501	PSAL 440−443	43,58,59
Parvibaculum lavamentivorans monooxygenase	PlBVMO	*Parvibaculum lavamentivorans*	A7HU16	6JDK	67	340	W 504	PSGF 443−446	60
polycyclic ketone monooxygenase	PockeMO	*Thermothelomyces thermophila* ATCC 42464	G2QA95	5MQ6	133	426	Y 600	S--Q 536−537	47
steroid monooxygenase	STMO	*Rhodococcus rhodochrous*	O50641	4AOS, 4AOX, 4AP1[b], 4AP3[b]	71	342	W 506	PSVL 445−448	61

[a]D: active-site aspartate, R: active-site arginine, Ω: active-site aromatic residue, bulge: active site insertion loop. [b]Mutated variant. [c]Crystallized in complex with an active-site ligand.

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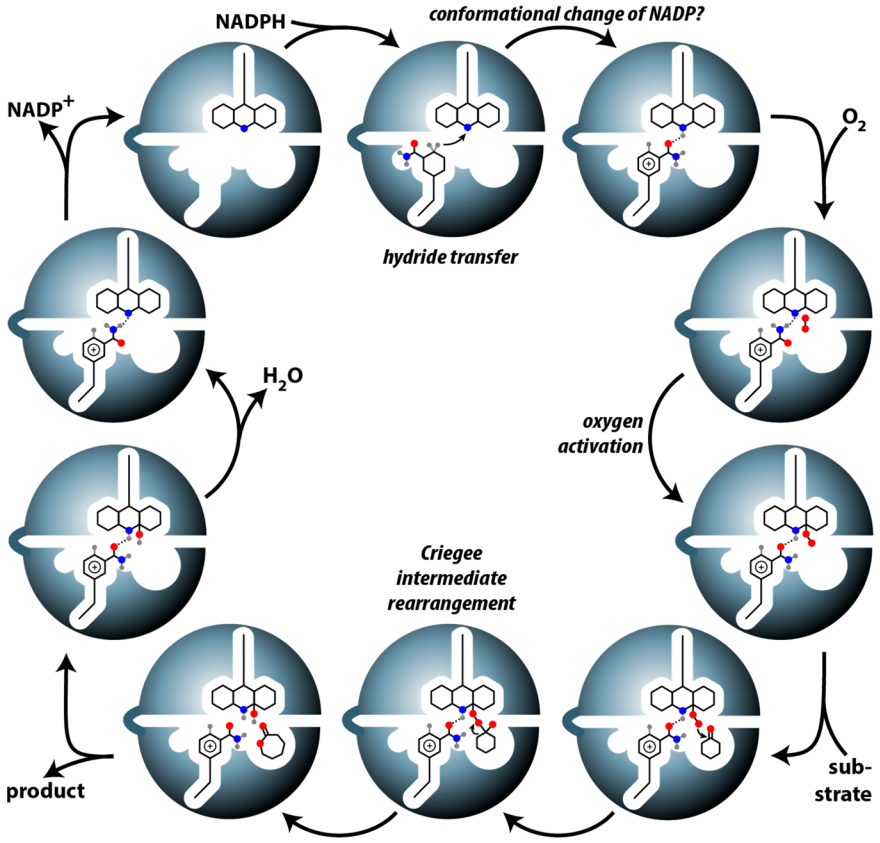
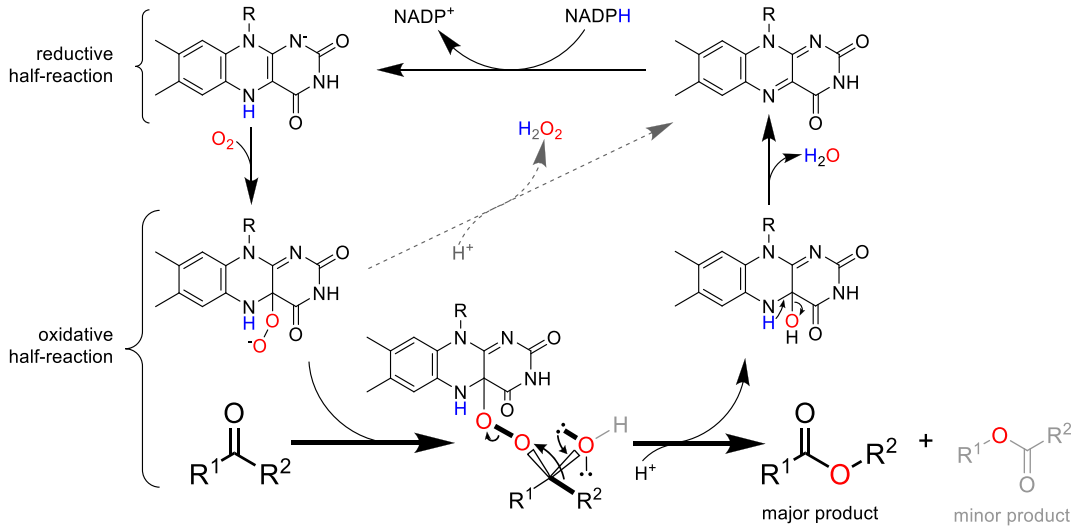
Scheme 1. Overall Catalytic Cycle of BVMOs Involving Various Redox States of the Flavin and Nicotinamide Cofactors[a]



[a]Important atoms are marked by red (oxygen), blue (nitrogen), or gray (hydrogen) circles.

Scheme 2. Reaction Mechanism of BVMOs[a]



[a]The flavin catalytic cycle consists of two half-reactions and ketone oxidation is catalyzed by a peroxyflavin, unless hydrogen peroxide loss causes an uncoupled NADPH oxidation (gray dashed arrow). The transformation from a ketone to an ester traverses through a regioselectivity-determining intermediate. Bond migration is dependent on the *anti*-periplanar alignment (indicated by thick bonds) of the migrating bond with the peroxy bond and one of the lone pairs on the former carbonyl oxygen. While protonated in the chemical Baeyer–Villiger reaction, this oxygen is, however, thought to be deprotonated in enzyme flavin intermediate (indicated in gray).

decay was observed in the absence of the cofactor.[62,66,76] Crystal structures and quantum mechanics calculations[77] indicate that the $NADP^+$ amide oxygen establishes a crucial hydrogen bond to the hydrogen of the flavin's N5 (Scheme 3). It is assumed that this stabilization prevents uncoupling by thwarting the otherwise quickly occurring proton transfer to the peroxy group and subsequent H_2O_2 elimination.[78] An active-site arginine, whose mutation abolishes Baeyer–Villiger activity,[79] was shown to be essential for the formation, but not for stabilization of the peroxyflavin.[76] The arginine ensures, however, peroxyflavin

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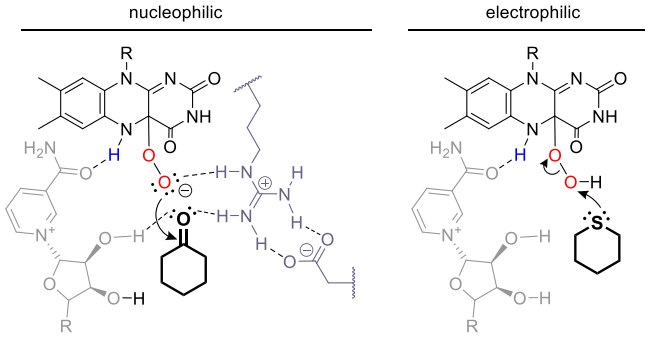
Scheme 3. Proposed Mechanism for Enzyme Catalyzed Oxidations[a]



[a]In the canonical, nucleophilic mechanism, the peroxyflavin attacks the substrate carbonyl. An active site aspartate increases the basicity of a neighboring arginine, which thus ensures deprotonation of the peroxyflavin. The arginine also activates the substrate ketone, supported by the 2′ OH of the ribose of $NADP^+$. In contrast, in the electrophilic mechanism a supposed hydroperoxyflavin reacts with the lone pair of a nucleophilic heteroatom.

deprotonation, supported by a nearby aspartate that increases the arginine's nucleophilicity (Scheme 3).[77] If a suitable ketone substrate is available, the next canonical step is the nucleophilic attack on the carbonyl group. In BVMOs, the proper positioning of the substrate is thought to be aided by a hydrogen bond between the 2′ OH group of the $NADP^+$ ribose and the carbonyl oxygen (Scheme 3).[77] The chemical Baeyer–Villiger reaction was already for a long time assumed to proceed via an intermediate whose nature initially caused some debate. Isotopic labeling experiments[80] eventually gave conclusive evidence for the pathway suggested by Rudolf Criegee,[81] in whose honor the tetrahedral intermediate was subsequently named. Although not directly observable, several computational studies support this mechanism.[82−85] Very recently, experimental evidence was provided from a stereoelectronic trap for the intermediate, using synthetic endocyclic peroxylactones.[86] In BVMOs, a flavin Criegee intermediate was also never observed, but in the absence of any counter-evidence, it is generally accepted that here the flavin and substrate also form an addition product, and computational studies support this theory.[77,87] The product then results from a concerted subsequent migration step, in which the weak O–O bond is heterolytically cleaved, while a new C–O bond is formed. The rearrangement proceeds with retention of configuration[88,89] and is often rate-determining for the chemical reaction, although both experimental[29] and theoretical[82,90] evidence indicate that the kinetics can change depending on the substituents, pH, and solvent. The regioisomeric outcome of the reaction is generally predictable and governed by a combination of influencing parameters. First, because of the positive charge developing on the migrating carbon in the transition state, the more electronegative carbon, which is better able to accommodate this charge, is more apt to migrate.[91] Thus, carbons with electron-donating substituents and those allowing resonance stabilization migrate better than methyl groups and electron withdrawing substituents.[29] Second, the C–C bond migrates preferentially when it is *anti*-periplanar to the peroxy O–O bond (Scheme 2), a condition known as the primary stereoelectronic effect.[92] Its influence on determining migration is apparently more significant than the migratory aptitude. This was concluded from the observation that a less-substituted bond migrates when forced into an *anti*-periplanar conformation in a restrained bicyclic Criegee intermediate.[93] A secondary stereoelectronic effect has also been postulated, requiring that one of the lone electron pairs of the hydroxyl group in the intermediate also needs to be *anti*-periplanar to the peroxy O–O bond (Scheme 2).[94] This effect only manifests in certain substrates, where substituents can sterically hinder the hydroxyl group rotation and presumably plays no role in enzyme catalysis, where the hydroxyl group is assumed to be deprotonated.[77] Lastly, the arrangement can be influenced by steric effects.[95,96] These may furthermore already affect the addition step, where the nucleophilic attack must occur from a favorable angle.[29,97] Steric control becomes most obvious in the enzymatic reaction, where intermolecular steric restraints can enforce an otherwise electronically prohibited pathway. It is for that reason that BVMO catalysis allows the synthesis of products, which are not accessible by chemical means (Figure 2).

While the peroxide-catalyzed reaction finishes under formation of the corresponding acid, the flavin can pick up a proton

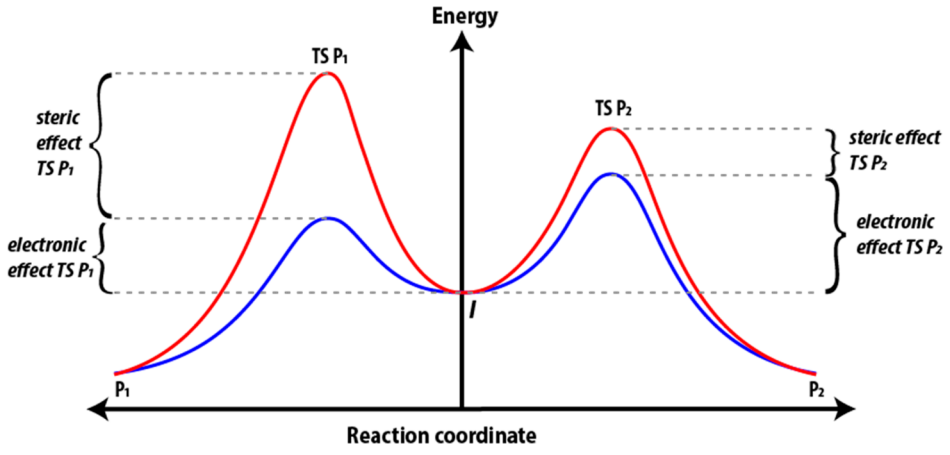


Figure 2. Simplified energy diagram depicting the electronic and steric effects affecting regioselectivity in BVMO reactions. In the Baeyer–Villiger reaction, an intermediate (*I*) is formed, which can undergo two varying migration pathways (Scheme 2), leading to two possible products (P_1 and P_2). In chemical catalysis, the predominant factors can collectively be called electronic effects, and the difference they exhibit on the energy of the two possible transition states, usually dictates the regioselectivity of the product (blue line). In enzyme catalysis, steric effects of active-site residues exhibit an additional force contributing to the overall energy of the transition states which can override the electronically favored pathway (red line).

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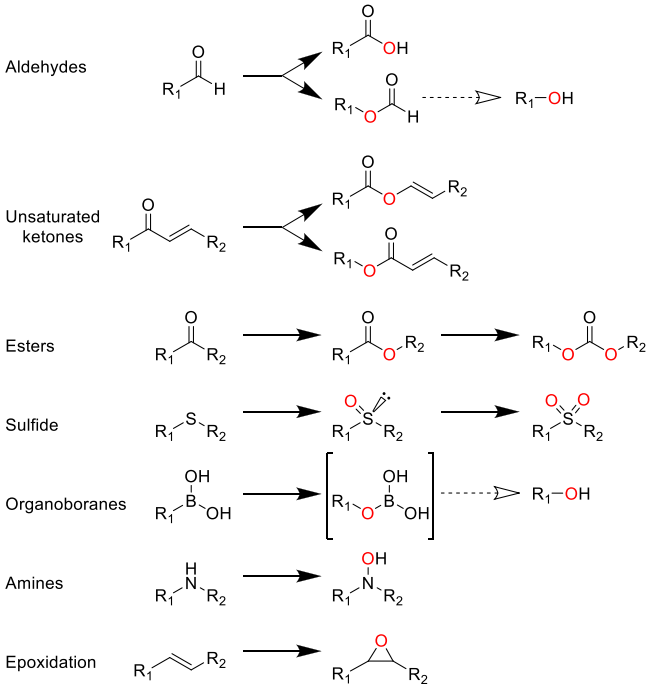
to form a hydroxyflavin, whose spontaneous dehydration reconstitutes the oxidized flavin.[67] It was suggested that this step is accelerated by a deprotonated active site residue with a pK_a of 7.3,[76] in line with the faster decay of this species at higher pH and the decreased overall reaction rates at low pH.[66,76] Before the enzyme can restart a new catalytic cycle, the oxidized nicotinamide cofactor needs to be ejected, and this step (or an associated conformational change) was found to be limiting to the overall reaction rate in CHMO.[66] In PAMO, the slowest catalytic step was not unambiguously identifiable, but may correspond to a conformational change prior to $NADP^+$ release.[76]

These findings entail two important and possibly conflicting conclusions: first, the two most detailed available studies on the mechanism of BVMO catalysis suggest that the enzymatic reaction is limited by a rate-determining step that is not involved in the chemical part of catalysis and therefore possibly substrate-independent. If this was generally the case, it could provide an explanation for the rather narrow range of maximal turnover rates observed for BVMOs with various substrates. Thus, reaction rates that are orders of magnitude higher than the currently known ones cannot be expected for any enzyme–substrate combination. However, this assumption is put in perspective by the second conclusion, which stems from the fact that (at least) the rate-determining step of catalysis appears to be nonidentical in CHMO and PAMO. If the two prototype enzymes differ in this crucial aspect, one cannot rule out that even other mechanisms dictate catalysis in other BVMOs. A generalization, therefore, may not be possible, and is furthermore impeded by the mechanistic variations in the chemical part of the reaction specified above, which always have to be considered to play a role on top of the enzymatic peculiarities.

PROMISCUOUS CATALYTIC ACTIVITIES

In addition to the canonical ketone oxidation, BVMOs also are able to perform a range of promiscuous catalytic activities (Scheme 4). Well-established and mechanistically analogous to the canonical reaction are BVMO oxidations of aldehydes,[98−103] including furans.[104] This reaction yields acids upon hydrogen migration, or otherwise (often unstable) formates. Although reactions with unsaturated ketones supposedly should also proceed identical in mechanism, most BVMOs show no reactivity with these weaker electrophiles. The transformation is also challenging chemically, where side reactions such as epoxidations frequently occur, and otherwise invariably enol esters are formed (i.e. oxygen insertion occurs toward the double bond).[105] Recently, two bacterial BVMOs were reported that can convert several cyclic α,β-unsaturated ketones.[106] Interestingly, the two enzymes reacted regiodivergently in some cases, which allowed the selective synthesis of both ene- and enol lactones. Although the crystal structure of the preferentially enol ester-forming enzyme—a BVMO from *Parvibaculum lavamentivorans*—has recently been solved, a structural explanation for its unusual reactivity has yet to be provided.[60] Only two other unsaturated ketones were reported to be accepted by BVMOs before: a substituted cyclopentenone, converted to the corresponding ene lactone by CPMO,[107] and pulegone, a cyclohexanone derivative with a double bond outside the ring on the α carbon, for which activity was reported with monoterpene ketone monooxygenase (MMKMO),[108] and cyclopentadecanone monooxygenase (CPDMO).[55] The three enzymes involved in campher degradation in *Pseudomonas putida*—2,5-diketocamphane 1,2-monooxygenase (2,5-DKCMO), 3,6-diketocamphane 1,6-monooxygenase (3,6-DKCMO) and OTEMO[109]—were also reported to convert several cyclopentenones and cyclohexenones. The results were questioned by the Alphand group, however,[106] although OTEMO's natural substrate is assumed to be a cyclopentenone derivative.[109,110] Conversion of a linear α,β-unsaturated ketone to the ene ester has been shown for the Baeyer–Villiger reaction-catalyzing human FMO5.[111] Oxidation of esters, which bear an even less electrophilic carbonyl, has been reported for a single BVMO, which is able to catalyze first the ketone oxidation and subsequently further converts the ester to its carbonate.[112]

Scheme 4. Non-Canonical Oxidation Reactions Catalyzed by BVMOs[a]



[a]Solid arrows represent enzymatic catalysis; a dashed arrow indicates spontaneous reaction.

Similarly to peroxides,[113] BVMOs were early found to promiscuously catalyze heteroatom oxidations as well.[98,114] Sulfoxidations are particularly well studied and many enzymes produced sulfoxides with high enantioselectivity.[103,115−125] Several existing patents describing the use of BVMOs for selective sulfoxidations emphasize the commercial potential.[126−128] Other reactions include oxidations of amines,[40,102,129,130] boron,[98,131,132] and selenium.[98,133,134] A single report of phosphite ester and iodine oxidation yet awaits further exploration,[98] as do the few reports of epoxidations catalyzed by BVMOs.[135,136] An entirely different approach to induce promiscuous catalytic activity is the use of BVMOs under anaerobic conditions to prevent peroxyflavin formation. Recent results with AcCHMO suggest that the so-stabilized reduced flavin can catalyze reductions, allowing tunable, stereoselective ketoreductase-like reactions.[137]

In contrast to the nucleophilic species required for the Baeyer–Villiger reaction, S-, N-, Se-, P-, and I- oxygenation require an electrophilic, protonated peroxyflavin. In line with the mechanism found for class A flavoprotein monooxygenases,[138] this hydroperoxyflavin was suggested to form in BVMOs, and an

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Table 2. Classification of Baeyer−Villiger Biocatalysts

group	flavoprotein subclass	hydride donor	prosthetic group	components[a]	prototype protein
type I BVMOs	B	NADPH	FAD	α	PAMO[43]
type II BVMOs	C	NADH	FMN (substrate)	$\alpha + \beta$	3,6-DKCMO[167]
type O BVMOs	A	NADPH	FAD	α	MtmOIV[168]
type I FMOs	B	NADPH	FAD	α	HsFMO5[111]
type II FMOs	B	NAD(P)H	FAD	α	RjFMO-E[160]

[a]α: Encoded by a single gene, $\alpha + \beta$: Encoded by multiple genes (monooxygenase and a reductase component).

apparent pK_a for the formation was determined to be 8.4 in CHMO.[66] However, as the protonated species in CHMO was not able to perform sulfoxidations, the results are not fully conclusive, and it was suggested that some protein conformational change is involved.[139] For PAMO, sulfoxidation enantioselectivity seems to depend on the protonation state of the peroxyflavin and the crucial[76,79] active site arginine;[140] and mutation of the arginine abolished both Baeyer−Villiger as well as sulfoxidation activity.[76] One study with CHMO, however, seemed to indicate that its heteroatom oxidation activity does not depend on the arginine, as the mutation to alanine or glycine yielded variants with retained S- and N- oxidation activity.[141] In this scenario, the loss of arginine could have two counteracting effects: as quantum mechanics studies suggest that a nearby aspartate protonates the arginine and this stabilizes the negatively charged, deprotonated peroxyflavin,[77] the arginine mutation could favor hydroperoxyflavin formation and thus the electrophilic mechanism. Contrarily, arginine loss decreases the overall reaction rate as the residue also promotes the reductive half-reaction and the rate of (hydro)peroxyflavin formation.[76,142] Interestingly, the substitution of a highly conserved aromatic residue with arginine was found in two independent studies that screened for variants with increased sulfoxidation activity.[42,127] In most BVMOs, this residue is a tryptophan that hydrogen bonds to the 3′ OH of the NADP ribose. Considering the enzyme's tolerance of other aromatic residues at this position,[143] this interaction is likely not influencing the electronics at the 2′ OH, which critically hydrogen bonds to the substrate carbonyl to activate it for nucleophilic attack (Scheme 3).[77] Rather, a mutation to arginine could push the positively charged coenzyme, possibly causing a disruption of the hydrogen bond to the substrate. Instead, the group might come closer to the peroxyflavin and cause its protonation; this mechanism would favor the electrophilic route and seems to be the mode of action in the closely related N-hydroxylating monooxygenases.[144]

VARIETY OF BVMOS

In the quest of discovering useful biocatalysts, many studies aimed to identify enzymes displaying features such as high regio- or enantioselectivity, showing a broad or a synthetically interesting substrate scope, lacking substrate or product inhibition and having high stability in typical process conditions. The classic ways to obtain novel efficient biocatalysts are mutagenesis on well-known catalysts and the exploitation of genome sequence databases, which are a rich and largely untapped resource for enzymes with attractive biocatalytic characteristics and novel chemistries.

BVMO Classification. Considerable research has been performed on BVMOs using comparative sequence analysis. Using a curated, representative sequence set, one study suggested that a BVMO gene was already present in the last universal common ancestor.[145] This study also found that there is no conclusive evidence that phylogenetic BVMO subgroups share biocatalytic properties, although this frequently has been and continues to be suggested in literature.[47,146,147] In the last decades, many BVMOs, both prokaryotic and eukaryotic, have been described, and approximately a hundred representatives were cloned and recombinantly expressed. In many cases, the natural role of those BVMOs could not be identified. In other cases, BVMOs were shown to be involved in the biosynthesis of secondary metabolites such as toxins,[148−152] or antibiotics.[153] While these enzymes often seem to be rather substrate specific, several BVMOs from catabolic pathways, involved in the degradation of cyclic aliphatics, for example,[38,154−156] can convert a larger range of substrates. Together with the structurally very similar N-hydroxylating- and flavin-containing monooxygenases, BVMOs have been classified as belonging to the class B of flavoprotein monooxygenases.[49] Recently, another sister group has been added—YUCCAs,[157] which are plant enzymes involved in auxin biosynthesis and shown to catalyze a Baeyer−Villiger-like reaction.[158] Some FMOs, including the human isoform 5,[111] were also found to catalyze Baeyer−Villiger reactions,[159] and it was suggested that these enzymes form a particular subgroup, classified as class II FMOs.[160] Their relaxed coenzyme specificity[161] enables interesting application opportunities.[162] Structurally largely unrelated are a few Baeyer−Villiger reaction-catalyzing enzymes found in class A[163] and C flavoprotein monooxygenases,[164] which otherwise comprise the aromatic hydroxylases and luciferases, respectively[49](Table 2). Cytochrome P450 monooxygenases, of which some can catalyze Baeyer−Villiger reactions,[165,166] are entirely unrelated and employ heme cofactors instead of flavins.

Many Baeyer−Villiger monooxygenases have been discovered and characterized by genome mining.[153,169−172] Instead of trying to be comprehensive, this Review will focus on some examples we believe are worthwhile to examine deeper (Figure 3, Table 3). From these proteins, most are type I BVMOs, which are encoded in a single gene and belong to the class B flavoprotein monooxygenases.[49] Several residues in BVMOs are highly conserved and useful for the identification of type I BVMOs. There are two specific sequences described: FxGxxxHxxxW[P/D][173] and [A/G]GxWxxxx[F/Y]P[G/M]-xxxD.[172] A modification to the short BVMO fingerprint was suggested (FxGxxxHTxxW[P/D]);[174] however, this consensus proved to be only partially conserved in a more divergent data set of sequences.[145] These motifs are flanked by two Rossmann fold domains harboring a GxGxx[G/A] motif required for tight binding of the two cofactors. In some cases, minor deviations from the consensus for the nucleotide binding sequence have been reported (MoxY, CPDMO).[155,175] Although the exact functional role of the fingerprint residues is not completely clear, the long consensus sequence entails the conserved active-site aspartate, while the short fingerprint is related to the linker connecting the FAD and NADP-binding domains.[43,59] As a common feature, type I BVMOs share the strict dependence on

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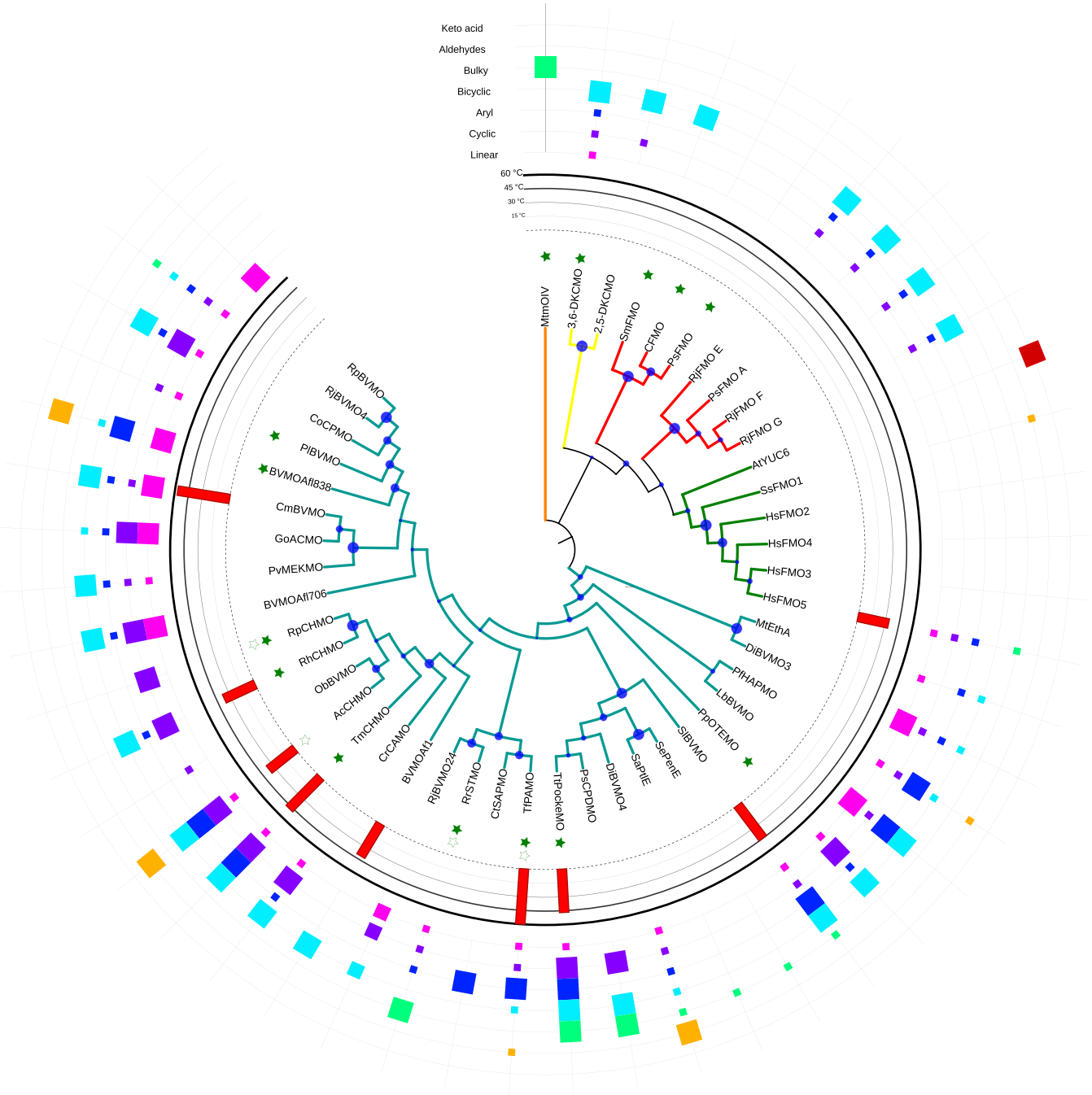

Figure 3. Cladogram analysis of BVMOs examples. The color of the clade represents the flavoprotein group to which the respective BVMOs belong (cyan for type I BVMOs, yellow for type II BVMOs, orange for type O BVMOs, green for type I FMOs, and red for type II FMOs). A star indicates the availability of crystal structures, in green for wild type and white for mutant. The bar chart shows the melting temperature. The outside rings represent the acceptance for different ketone substrates. Note that this only represents substrates that have been tested, while the actual scope might be (much) larger. The species and codes are listed in Table S1.

FAD as a tightly bound prosthetic group and NADPH as electron donor, with the exception of MekA from *Pseudomonas veronii* MEK700, which seems to accept either NADH or NADPH.[176] The preferred host for producing recombinant BVMOs, has been *Escherichia coli*, which does not contain a native homologue itself. BVMOs can also be directly applied in whole-cell conversions, as demonstrated in many reports focusing on valuable bioconversions (see section 'Biotechnological application'), but more detailed characterizations such as kinetic studies often use purified enzymes. Although some homologues show very high expression levels, *E. coli* may not be able to provide the cofactors in the necessary quantities,[177] thereby negatively affecting stability.[178] This effect is assumed to be even more critical when BVMOs are to be applied in in vivo cascades with other redox enzymes.[179] An additionally complicating factor in whole cell conversions is oxygen supply, which limits the reaction at high biomass concentrations.[180] When BVMO homologues with interesting biocatalytic properties were found to express poorly, several approaches to improve functional expression and stability were explored. Besides optimization of the expression conditions (cultivation temperature and time, induction method) and the more and more

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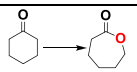
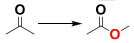
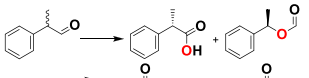
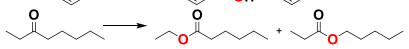
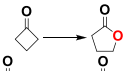
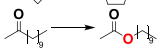
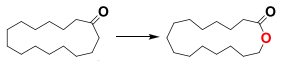
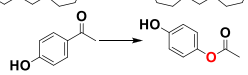
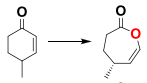
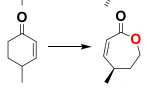
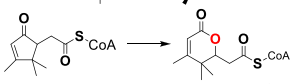
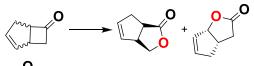
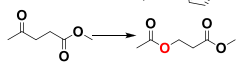
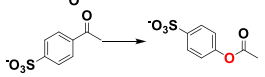
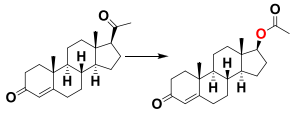
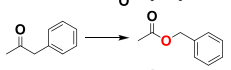
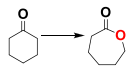
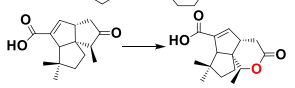
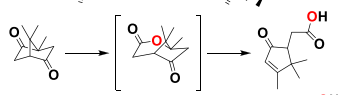
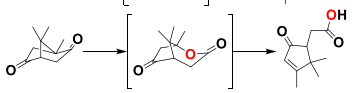
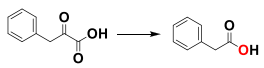
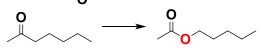
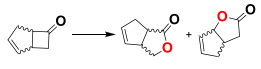
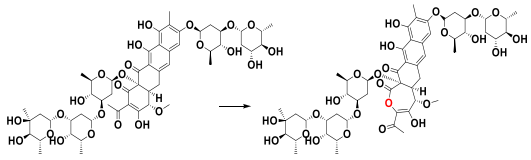
Table 3. Prototype Reactions of Baeyer−Villiger Monooxygenases[191192]

Name	Substrate	Prototype reaction	k_{cat} [s^{-1}]	K_m [μM]	Ref
AcCHMO	cyclohexanone		6.0-39	3-9	46, 182-183
GoACMO	acetone		1.4	170	184
BVMO4	2-phenyl-propionaldehyde		n.d.	n.d.	185
BVMOAfl838	3-octanone		6.6	170	51
CAMO	cyclobutanone		6.8	7	186
CmBVMO	2-dodecanone		0.4	4	187
CPDMO	cyclopentadecanone		4.2	6.0	155
HAPMO	4-hydroxy-acetophenone		10-12	9-40	103, 188
ObBVMO	4-methylcyclohex-2-en-1-one		n.d.	n.d.	106
PlBVMO	4-methylcyclohex-2-en-1-one		n.d.	n.d.	106
OTEMO	2-oxo-Δ^3-4,5,5-trimethyl-cyclo pentenylacetyl-CoA		4.8	18	57
PockeMO	bicyclo[3.2.0] hept-2-en-6-one		3.3	400	47
RpBVMO	methyl levulinate		1.5	350	189
SAPMO	4-sulfoacetophenone		2.9	60	190
STMO	progesterone		0.7	85	191-192
TfPAMO	phenylacetone		1.9-3	60-80	169, 193
TmCHMO	cyclohexanone		2.0	<1	46
PtlE	1-deoxy-11-oxopentalenate		n.d.	n.d.	153
2,5-DKCMO	2,5-diketocamphane		n.d.	n.d.	164
3,6-DKCMO	3,6-diketocamphane		n.d.	n.d.	164
AtYUC6	phenylpyruvate		0.31	43	158
HsFMO5	2-heptanone		n.d.	n.d.	194
RjFMO-E	bicyclo[3.2.0] hept-2-en-6-one		2.0-4.3	3	161-162
MtmOIV	premithramycin B		0.7	70	195

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common use of synthetic genes with host-optimized codons, fusion approaches with soluble tags are popular countermeasures. One study also coexpressed molecular chaperones with a BVMO from *P. putida* and found that optimal results rely on their distinct expression levels.[181]

Eukaryotic Type I BVMOs. Baeyer−Villiger oxidations have frequently been demonstrated in physiological studies.[196−200] BVMO genes were described as scarce in microorganisms,[173] though in fact they exhibit an uneven genomic distribution.[201] While bacterial BVMOs are most abundantly found in actinomycetes, there is also a high prevalence in some filamentous fungi. Particularly, BVMOs were found in Basidiomycota, Zygomycota, and the Ascomycota, where they are especially abundant in the *Aspergillus* genus.[145,146] Until recently, most of the research with isolated enzymes investigated prokaryotic BVMOs—possibly because of the easiness to work without the splice components of eukaryotes or to avoid problems with rare codons. One of the first type I BVMOs obtained from a fungus was steroid monooxygenase from *Cylindrocarpon radicicola* ATCC 11011 (CrSTMO), which was purified from cells grown in the presence of progesterone.[202] Although several fungi with Baeyer−Villiger activities were described, it was only in 2012 when the first recombinant fungal BVMO was expressed by the group of Bornscheuer.[186] This enzyme comes from the same ascomycete as CrSTMO. This fungus is also described to metabolize cyclohexanone as a carbon source, and this ability was linked to the presence of a second BVMO, identified as cycloalkanone monooxygenase (CAMO). CAMO shows 45% sequence identity with AcCHMO and exhibits a broad substrate scope, among which cycloaliphatic and bicycloaliphatic ketones showed the highest activities. However, its thermostability is quite poor, and with 28 °C, the temperature for 50% residual activity after 5 min of incubation is considerably lower than that of AcCHMO (36 °C).[203] BVMOAf1 from the fungus *Aspergillus fumigatus* Af293 was described one year later.[204] This BVMO stems from a pathogenic organism that is known to be a source of biocompounds such as helvolic acid and fumagillin, in whose biosynthesis the enzyme could be involved. Its activity was found to be relatively low, with maximal rates of catalysis around 0.5 s^{-1}; however, high enantioselectivities in the oxidations of thioanisole, benzyl ethyl sulfide and bicyclo[3.2.0]hept-2-en-6-one were observed. This enzyme exhibits relatively high thermostability: while the highest activity was recorded at 50 °C, the T_m was 41 °C. In addition, after 1 h of incubation in buffers containing 5% of various cosolvents, its activity remained without significant loss. Four other enzymes were discovered from *A. flavus* NRRL 3357 (BVMOAfl210, 456, 619 and 838).[205] From those, BVMOAfl838 displayed a high conversion of aliphatic ketones, but it was unable to convert most of the cyclic ketones tested. BVMOAfl838 later was the first reported crystal structure of a fungal type I BVMO.[51] The enzyme showed an optimal temperature of approximately 40 °C, but was rapidly inactivated at that temperature, displaying a half-life of only 20 min. The structure could not be cocrystallized either with the nicotinamide cofactor or with the substrate and showed a global fold similar to other described BVMOs. Near to the supposed substrate entry channel is a mobile loop that presents a lysine (K511). This residue was suggested to be proximal to the 2′-phosphate of NADPH, and the K511A mutant exhibited a higher uncoupling. Later, more BVMOs from *Aspergillus* were characterized: BVMOAfl706 and BVMOAfl334 (~45% amino acid sequence identity), which converted a range of cyclic and substituted cyclic ketones and showed the highest conversions and k_{cat} values of 4.3 s^{-1} and 2 s^{-1} for cyclohexanone, respectively.[206] Interestingly, no substrate inhibition was observed for BVMOAfl706 with cyclohexanone using concentrations up to 30 mM. In contrast, AcCHMO, shows a K_i of approximately 35 mM[207,208] Subsequently, a study tried to exploit BVMOAfl706 in a cascade reaction for the lactonization of cyclohexanone, but the enzyme seemed to be responsible for the formation of an undesired side product.[209] The last fungal example is polycyclic ketone monooxygenase (PockeMO) from the thermophilic fungus *Thermothelomyces thermophila*, which was discovered and crystallized.[47] This fungus is known to efficiently degrade cellulose and derivatives from plant biomass. This enzyme presented high enantioselectivity for bicyclo[3.2.0]hept-2-en-6-one and displayed an unusually broad activity on several polycyclic molecules, hence its name. PockeMO exhibited the highest activity at 50 °C and a melting temperature (T_m) of 47 °C. As metabolically observed for fungi[196] and as was described for CrSTMO[202] and CPDMO,[210] PockeMO is able to regioselectively catalyze the D-ring oxidation of steroid substrates producing the normal lactone. Later, de Gonzalo analyzed the applicability of PockeMO for the synthesis of optically active sulfoxides and showed full conversion of thioanisole into the (*R*)-sulfoxide with excellent selectivity, while for other alkyl phenyl sulfides, a decreased activity and selectivity was observed.[117] Thermostable enzymes have also been found in photosynthetic organisms: CmBVMO from the red algae *Cyanidioschyzon merolae* and PpBVMO from the moss *Physcomitrella patens*.[187] They showed high thermostability, in particular CmBVMO, which displayed a T_m of 56 °C, whereas PpBVMO's T_m was 44 °C. Although their activity was comparatively low, with k_{cat} values in the 0.1−0.3 s^{-1} range, they could achieve modest conversions of cyclohexanone.

Prokaryotic Type I BVMOs. Among the many bacterial type I BVMOs described in the last years, there are several homologues of AcCHMO, as one goal was to identify a similar but more stable biocatalyst. One particular example is TmCHMO, which shows 57% sequence identity with AcCHMO.[46] This enzyme stems from *Thermocrispum municipale* DSM 44069, a thermophilic microorganism isolated from municipal waste compost. TmCHMO was described to efficiently convert a variety of aliphatic, cyclic, and aromatic ketones and was also able to oxidize prochiral sulfides. Interestingly, TmCHMO exhibits a T_m of 48 °C and presents stability against high temperatures and the presence of cosolvents. However, as AcCHMOs, this robust enzyme showed inhibition with high substrate concentrations.[208,211] Another newly described BVMO is BVMO4, identified from the genome of *Dietzia* sp. D5. This enzyme phylogenetically clusters with cyclopentadecanone monooxygenase (CPDMO).[212] BVMO4 displayed a broad substrate scope accepting different ketones and sulfides but showed low activity. Although BVMO4 converted alicyclic and aliphatic ketones only moderately, it was also studied for its activity with phenyl group-containing and long aliphatic aldehydes. With respect to the latter, BVMO4 showed high regioselectivity with for example octanal, decanal, and 3-phenylpropionaldehyde, and preferentially synthesized the respective carboxylic acid over the formyl ester. Albeit with rather poor selectivities, this was the only reported BVMO able to convert a 2-substituted aldehyde to the respective acid, which is a precursor of ibuprofen and derivatives.[185] An effort to improve the activity of BVMO4 with cyclohexanone by site saturation mutagenesis over 12 described hot spots was

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reported.[213] Its activity was successfully increased against cyclic ketones and the oxidation of cyclohexanone was improved. A thorough biochemical characterization was described for a BVMO active on small substrates, acetone monooxygenase (ACMO) from the propane-metabolizing organism *Gordonia* sp. TY-5.[184] ACMO converts small ketones such as acetone and butanone with k_{cat} values between 1.4−4.0 s^{-1}; but shows only modest stability, losing over 60% of the activity after 1 h incubation at 25 °C in buffer. This enzyme displayed a weaker affinity for bulkier substrates and NADPH. The latter was suggested to be caused by a diminished electrostatic interaction between the 2′-phosphate of the coenzyme and the protein due to a substitution of a usually conserved lysine[79] by histidine. Additionally, a monooxygenase from *Leptospira biflexa* that was phylogenetically distant from other well-characterized BVMOs was described by the group of Rial in 2017.[214] LbBVMO showed a broad substrate scope for acyclic, aromatic, cyclic, and fused ketones and allowed the highly regioselective conversion of aliphatic and aromatic ketones. For *Rhodoccocus jostii* RHA1, 22 BVMOs were found in the genome, which showed a diverse scope when tested against a large set of potential substrates including different ketones and sulfides.[147,172] From these enzymes, at least two are quite promiscuous regarding their substrate scope (RjBVMO4 and RjBVMO24), accepting the majority of the 25 tested compounds.

Furthermore, there a few well-described BVMOs from Pseudomonads, like HAPMO and OTEMO, from *P. fluorescens* ACB and *P. putida* NCIMB 10007, respectively.[110,188] The former has 30% sequence identity with AcCHMO and was studied for the oxidation of a wide range of acetophenones, such as 4-hydroxyacetophenone, 4-aminoacetophenone, and 4-hydroxypropiophenone. For these substrates, HAPMO has k_{cat} values between 10 and 12 s^{-1}. This enzyme has also been reported to catalyze the oxidation of fluorobenzaldehydes, aryl ketones, and sulfides.[100,118,215] OTEMO, conversely, is involved in the metabolic pathway of camphor and was described to oxidize the cyclopentanone derivative 2-oxo-Δ^3-4,5,5-trimethyl-cyclopentenylacetyl-CoA. While it exhibits a rate of 4.8 s^{-1} for its natural substrate, the free acid shows a rate 30 times lower than for the CoA ester.[57] OTEMO has been mostly studied for the conversions of substituted cyclohexanones, bicyclic ketones and terpenones.[57,109,216] Another BVMO from *Pseudomonas* is PpKT2440-BVMO from *P. putida* KT2440.[217] This enzyme showed acceptance for aliphatic ketones but exhibited low conversions for cyclic and aryl ketones. The highest levels of oxidation were reported for 2-, 3-, and 4-decanone (93−99% conversion using resting cells). Later, this enzyme was engineered for the whole cell biotransformation of ricinoleic acid into a precursor of polyamide-11 (nylon-11), achieving conversions of 85% and a product concentration of up to 130 mM.[218,219]

The latest example is a BVMO from *Rhodoccocus pyridinivorans* DSM 44555.[189] RpBVMO exhibited affinity for aliphatic methyl ketones and the highest activity on 2-hexanone (k_{cat} = 2 s^{-1}). RpBVMO was able to regioselectively convert hexanones, octanones, and methyl levulinate. The latter is a 2-ketone derived from renewable levulinic acid gained from biomass. Interestingly, the biocatalyst was used to fully convert 200 mM of this substrate to methyl 3-acetoxypropionate with a space-time yield of 5.4 g L^{-1} h^{-1}. The hydrolyzed product, 3-hydroxypropionate is a platform chemical used as sugar-derived building block for biodegradable polymer polyester synthesis and is an important intermediate in the nonpetrol-based production of a variety of bulk chemicals.[220]

Type II BVMOs. Type II BVMOs are categorized as class C flavoprotein monooxygenases, which are two-component monooxygenases. During the catalytic cycle, one component reduces FMN using NADH or NADPH as hydride donor. The flavin is then transferred by free diffusion to the second component, which uses reduced FMN as cosubstrate for oxygen activation.[221] This is biochemically interesting because the free reduced FMN could lead to nonselective reactions with molecular oxygen inside the cell.[222] This group is less studied than type I BVMOs, perhaps because of the higher convenience to work with only one component. In addition to the early confusion with the actual prosthetic group, it was previously mistakenly believed to be a flavoprotein using tightly bound FMN as a coenzyme and that was reduced in situ in the active site by NADH.[223] There are some examples of type II BVMOs related to the metabolic pathway of the racemic monoterpene camphor. In particular, the enzymes involved are 2,5- and 3,6-diketocamphane monooxygenases (2,5-DKCMO and 3,6-DKCMO). These proteins are encoded on the linear inducible CAM plasmid from *P. putida* ATCC 17453 and were named after their natural substrates.[223] The presence of two isoforms in the same plasmid was described for 2,5-DKCMO, one being localized 23 kb downstream and encoded on the opposite strand.[164] It was suggested that the high sequence identity between them is the result of a gene duplication event and a sequence divergence in the case of 3,6-DKCMO. 2,5-DKCMO and 3,6-DKCMO oxidize the third metabolic step of the catabolism of *rac*-camphor and are specific toward one enantiomer. They specifically act on 2,5 and 3,6-diketocamphene, respectively. In recombinant cells expressing the oxygenating subunit of 2,5 or 3,6-DKCMO, activity without a recombinant FMN reductase component was noticed, which was explained by the activity of native reductases from the host.[109] Later, Fred, a homodimeric reductase encoded in the chromosomal DNA of *P. putida* was suggested to be the bona fide reductase component for the three DKCMOs (3,6- and 2 isoforms of 2,5-DKCMO).[164] The complexes were tested against several substrates, exhibiting exclusive specificity for the natural substrate. Later, the structure of the oxygenating component of 3,6-DKCMO was solved in complex with FMN and showed a fold most similar to the bacterial luciferase-like superfamily.[167] The structure was somewhat controversial because of experimental discrepancies.[222,224] Other members of the type II BVMOs are luciferases from *Photobacterium phosphoreum* NCIMB 844 and *Vibrio fischeri* ATCC 7744.[225] These two-component bacterial luciferases catalyze Baeyer−Villiger reactions of 2-tridecanone, monocyclic and bicyclic ketones. In addition, it was suggested that an NADPH-dependent 6-oxocineole monooxygenase of *Rhodococcus* sp. C1 could also be part of this class.[226]

Type O BVMOs. The best-studied BVMO of the type O—for atypical or "odd" BVMOs—is MtmOIV from the soil actinomycete *Streptomyces argillaceus* ATCC 12956. The enzyme is a homodimer involved in the biosynthesis of mithramycin, an aureolic acid-like polyketide studied as an anticancer drug and calcium-lowering agent.[163,227] This enzyme does not have significant sequence identity with other well-described BVMOs, does not display the consensus motifs for type I BVMOs, and bears no structural resemblance with type I or type II BVMOs. This monooxygenase catalyzes the Baeyer−Villiger oxidation of one of the four rings of premithramycin B,

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forming the lactone, which is later converted to mithramycin DK. As other BVMOs, MtmoIV uses NADPH and FAD as hydride donor and prosthetic group, respectively. The enzyme belongs to the class A flavoprotein monooxygenases, and it has been suggested that their reaction requires a peroxyflavin intermediate for nucleophilic attack, even though class A flavoprotein monooxygenases classically form a hydroperoxyflavin and proceed through an electrophilic attack.[49] The crystal structure was solved in complex with FAD, and the active site contains an arginine residue (R52) over the isoalloxazine ring, which presumably stabilizes the negatively charged peroxyflavin and Criegee intermediates.[168] While classic BVMOs contain a positively charged arginine on the *re* side of the flavin, MtmOIV's R52 is on the *si* side. Structurally, MtmOIV is most similar to *para*-hydroxybenzoate hydroxylase as well as the glucocorticoid receptors (GR2) subclass of FAD-dependent enzymes.[168,228] Unsurprisingly, as MtmOIV catalyzes the oxidation of a bulky tetracyclic polyketide with deoxysugar modifications, it has a large binding pocket for the substrate, which may interact mostly by van der Waals and hydrophobic interactions.[195] Concerning the kinetic parameters, this enzyme displays relatively low activities in the presence of the natural substrate. Despite this, MtmOIV is interesting to investigate, as it might be a useful biocatalyst for the oxidation of analogues of premithramycin B and allow a synthetic route to new drugs.

Flavin-Containing Monooxygenases. Flavin-containing monooxygenases (FMOs), like type I BVMOs, are part of the class B flavoprotein monooxygenases and are described to catalyze the oxidation of "soft" nucleophilic heteroatoms in a broad spectrum of substrates.[229] FMOs are single-component enzymes, contain FAD as a prosthetic group, and have a preference for NADPH over NADH as FAD-reducing coenzyme.[49] For FMOs, two types have been described: type I FMOs are identified by the motif FxGxxxHxxx[Y/F], which is similar to the short consensus motif of type I BVMOs. Mammals, including humans, express five transmembrane FMO isoforms in a developmental-, sex-, and tissue-specific manner.[230] These enzymes are involved in the metabolism of xenobiotics such as drugs, pesticides, and certain dietary components.[111] While this group is described to oxidize mainly nitrogen and sulfur atoms, exceptions to this rule have been identified early on: for example, isoform FMO1 from pig liver was able to catalyze the Baeyer−Villiger oxidation of salicylaldehyde to pyrocatechol.[231] In addition, the human isoform FMO5, which expresses mostly in the small intestine, the kidney, and the lung and has been described to exhibit poor activities on classic FMO substrates, is also able to catalyze Baeyer−Villiger oxidations. The enzyme was recombinantly expressed, and converted preferentially aliphatic ketones, but also aldehydes and cyclic ketones with varying regioselectivity.[111] Consequently, it was proposed that HsFMO5 could act as a possibly undescribed detoxification route in human metabolism. In this regard, it is remarkable that the enzyme can convert, for example, nabumetone and pentoxifylline (two ω-substituted 2-ketone drugs) and also a metabolite of E7016—a potential anticancer agent.[194] On the other hand, HsFMO5 was also described to have a high uncoupling rate, constituting for 60% of the activity. This phenomenon was ascribed to a low C4α-(hydro)peroxyflavin stabilization because of a weaker interaction with $NADP^+$. Another group among the type I FMOs is formed by the YUCCAs,[145] which have a key role in the physiology of monocots and dicots plants. These enzymes catalyze a rate-limiting step in de novo auxin biosynthesis, an essential growth hormone and development regulator.[157,232] Notably, 11 of the 29 putative FMOs in *Arabidopsis thaliana* belong to the YUCCA family, and one of them, AtYUC6, was described to catalyze the decarboxylation of indole-3-pyruvate to the auxin indole-3-acetate.[158] A sequence similarity network shows that YUCCAs are more related to FMOs than to BVMOs, even though the predicted mechanism is more related to the latter. As in the reaction of BVMOs, catalysis proceeds through a Criegee intermediate with a nucleophilic attack by the C4α-(hydro)peroxyflavin followed by a decarboxylation step producing the auxin. For AtYUC6, as for HsFMO5, a short-lived C4α-(hydro)peroxyflavin intermediate was measured.[111,158] Additionally, a few enzymes that constitute the novel subclass of type II FMOs have been discovered in recent years. As the type I FMOs, this group can catalyze both heteroatom oxidations, as well as Baeyer−Villiger oxidations. Unlike the type I BVMOs, these enzymes cannot be identified by the long fingerprint sequence but contain two Rossman fold motifs and exhibit the type I FMO motif FxGxxxHxxx[Y/F][K/R] with a few substitutions: a histidine instead of [Y/F] and aspartate, proline, valine, or glycine instead of [K/R].[160,233] It was reported that these enzymes are promiscuous for the hydride donor, accepting either NADH or NADPH. This feature is attractive because the change of specificity for the cofactor of NADPH-dependent BVMOs is not a trivial task, as has been seen in studies of BVMO variants generated to identify residues related to the specificity for NADPH and the improvement of NADH catalytic efficiency.[79,234,235] At present, there are some attempts to investigate this new group in more detail. Enzymes from *Pseudomonas stutzeri* NF13 (PsFMO), *Cellvibrio* sp. BR (CFMO), and *Stenotrophomonas maltophilia* PML168 (SmFMO) were studied. Although the kinetic parameters, conversion yields, enantioselectivities and substrate scope turned out to be poor, SmFMO displayed similar activities either with NADH or NADPH. For SmFMO the K_m for the prototypic substrate bicyclo[3.2.0]hept-2-en-6-one was 40 times lower with NADH than with NADPH, and the conversion of the substrate was also considerably higher (90% vs 15%, respectively).[159] SmFMO was cocrystallized in complex with FAD, and it was suggested that the promiscuity is linked to the replacement of Arg234 and Thr235 as occurring in MaFMO—a related type I FMO from *Methylophaga aminisulfidivorans* —by a glutamine and a histidine (Gln193 and His194). However, the double mutant did not radically affect the cofactor specificity in SmFMO, but the single mutant H194T caused a switch in cofactor preference from NADH to NADPH (mostly by reducing the $K_{m,NADPH}$).[236] This effect was suggested to be related to the interaction of T235 with the ribose 2′-phosphate oxygen in MaFMO. Later, two novel proteins were found with variations of MaFMO's R234 and T235: CFMO and PsFMO, which share 58% and 61% sequence identity with SmFMO, respectively.[237] These enzymes were also described to accept NADH as a cofactor but were mostly studied for asymmetric sulfoxidations. Another subgroup of type II FMOs, which features sequence alterations like an extension in the N-terminus, showed higher conversions and broader substrate scope for ketones. These include the FMOs from *R. jostii* RHA1, RjFMO-E, F and G,[160] and PsFMO-A, B and C from *Pimelobacter* sp. Bb-B.[233] RjFMO-E, F, and G were found to be able to convert the classic substrate bicyclo[3.2.0]hept-2-en-6-one and cyclobutanones, but displayed only modest enantioselectivities and performed poorly in catalyzing the oxidation of phenylacetone. RjFMO-E displayed a higher affinity

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for NADPH, but also the affinity for NADH is in the micromolar range. Interestingly, the k_{cat} for bicyclo[3.2.0]hept-2-en-6-one with NADH is higher than that with NADPH (4.3 vs 2.7 s^{-1}) and almost 80 times higher than the reported k_{cat} for SmFMO.[159,160] Finally, PsFMO A-C, three enzymes from a hydrocarbon-degrading bacterium were studied. These proteins show a sequence identity of 29−35% with RjFMO-E. PsFMO-A displayed the widest substrate scope, and like the FMOs from *R. jostii*, the highest activities were obtained with the ketones camphor and bicyclo[3.2.0]hept-2-en-6-one. High conversions were observed, but the enantioselectivities were only high for the normal lactone (>99% ee for the normal lactone and 57% ee for the abnormal lactone). More studies are expected for this class of enzymes, as their cofactor promiscuity constitutes a big potential in future biocatalysis. It remains unknown whether or not NADH can fulfill the dual catalytic role described for NADPH in classical BVMOs—as hydride donor and stabilizer of the hydroperoxide flavin.

■ ENZYME ENGINEERING

Besides the usefulness in gaining mechanistic insights, mutagenesis in BVMOs has been used to deliberately alter various enzyme properties. A large body of work has focused on altering substrate scope and selectivities. These studies have often focused on what have become the two prototypes, AcCHMO and PAMO. The two enzymes can be seen as the "yin and yang" of BVMO research: AcCHMO was discovered early on, but no structure was available until very recently a 10-fold mutant was crystallized; it acts on a broad range of substrates and often shows high stereoselectivity, but with a T_m of 37 °C,[182] it is marked by poor stability. On the contrary, PAMO was discovered much later, but the crystal structure was solved immediately; its substrate scope is limited to aromatic compounds, and its stereoselectivity is often poor, but with a T_m of 61 °C,[238] it is very stable. For these reasons mutagenesis in PAMO focused on substrate selectivity engineering and in AcCHMO at manipulating product specificity and thermostability.

Efficient protein engineering of BVMOs became possible after recombinant strains of *E. coli*[239] and yeast[240] were available. In the absence of a crystal structure, early mutagenesis experiments focused on investigating the functional role of conserved residues (Figure 4).[173,188,241] In recognition of their potential for application, one of the first attempts of rational protein engineering in BVMOs was targeting their dependency on NADPH, which is more costly and less stable than NADH. By changing conserved basic residues close to the Rossmann motif, a lysine in 4-hydroxyacetophenone monooxygenase (HAPMO) was identified to strongly determine NADPH specificity.[79] Mutagenesis to phenylalanine decreased the K_m for NADH ~5-fold, while mutagenesis to alanine in AcCHMO decreased it ~2-fold. A later study in PAMO did not observe the same effect upon mutating the corresponding residue, but identified a nonconserved histidine, whose mutation to glutamine decreased the K_m for NADH ~4-fold.[235] More recently a larger set of mutations was probed in AcCHMO, but the best mutant decreased the $K_{m,NADPH}$ only ~2.5-fold.[234] The mutations of the various studies also increased the maximal turnover rate with NADH, leading to a moderate increase in catalytic efficiencies, and decreased the specificity for NADPH (Table 4). The latter effect was especially dominant in AcCHMO when a substitution of a conserved [S/T] with glutamate was combined with targeting the previously found lysine. The resulting mutant was still so poor with NADH, however, that bioconversions of 5 mM of AcCHMO's native substrate, cyclohexanone, was only possible when using stoichiometric amounts of the cofactor.[234] The fact that the switch of cofactor specificity—while often successful in other enzyme classes[242,243]—was largely unsuccessful in BVMOs, highlights the complex role of NADP in class B monooxygenases. It is now well-known that NADP fulfills at least a dual function in catalysis: flavin reduction and peroxyflavin stabilization.[50] In doing so, the cofactor likely undergoes conformational changes whose stabilization and interchange need to be in a balance that is easily impaired by mutagenesis. Though unclear, it seems likely that the same or similar considerations apply to Baeyer−Villiger reaction-catalyzing FMOs, where an example of the reverse engineering from NADH to NADPH has been described for an FMO from *Stenotrophomonas maltophilia*.[236] While the wild-type enzyme accepts both cofactors with slight preference for NADH, a mutant with a ~5-fold higher catalytic efficiency with NADPH was generated, and its structure was solved.

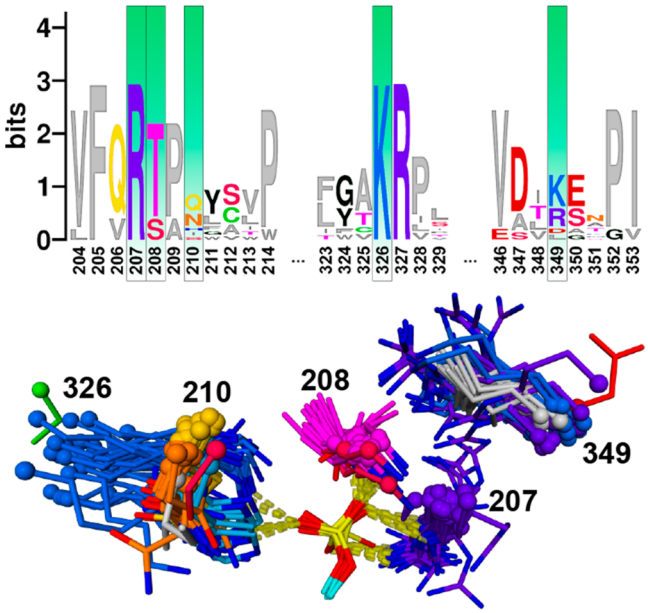

Figure 4. NADPH specificity. Top: the weblogo shows the sequence conservation at relevant residues (numbering of AcCHMO), highlighted with a green box. Bottom: all available structures of BVMOs superimposed, and the residues surrounding the phosphate group of NADPH are shown (corresponding to the highlighted residues in the top part). Residues are shown as sticks, α carbons are marked as a ball, and the coloring of carbons is according to the color scheme in the top. Hydrogen bonds are shown as yellow dotted lines.

An even more important factor for application is catalyst stability. For many enzymes, the main focus of attention is operational stability—as storage stability is more easily addressed, because most enzymes can be kept frozen in solution for up to years or otherwise be kept as lyophilized powders. For BVMOs, one study found that lyophilization in the presence of sucrose aids in preserving catalytic activity.[244] In the course of this work, the generally very poor stability of AcCHMO was also quantified: upon storage at 4 °C, the enzyme lost half of its activity after 72 h. Being a well-known phenomenon, the challenge of overcoming its instability has been an aim of a number of studies. Assessing their successes, however, is complicated because of the use of nonstandardized assays, and a certain lack of agreement in the field on how thermostability

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Table 4. Enzyme Variants Generated to Switch Cofactor Specificity

enzyme	mutation(s)	fold increase $k_{cat,NADH}$	fold decrease $K_{m,NADH}$	fold increase $k_{cat,NADH}/K_{m,NADH}$	fold decrease NADPH/NADH	ref
HAPMO	K439F	1.4	4.8	6.7	410	79
PAMO	H220Q	6.9	3.7	3.3	8.6	235
AcCHMO	K326A	0.4	1.8	0.7	58	79
AcCHMO	S186P/S208E/K326H	3.1	2.5	8	1900	234

best is measured and compared. Symptomatically, when a recent study looked at literature data on the half-life of wild-type AcCHMO at 25 °C, it was noted that the reported values span more than an order of magnitude.[178] In analogy to the uncertainties associated with assays determining temperature-dependent enzyme activities,[245] the authors argue that the commonly used spectrophotometric cuvette assays are prone to produce unreliable results. Applying the assay nonetheless, this study subsequently investigated the effects of additives on AcCHMO's stability and linked it to cofactor concentration and presence of reactive oxygen species (ROS). Specifically, a high excess of NADPH, but not $NADP^+$ increased the enzyme's half-life, and so did addition of FAD and ROS-scavenging enzymes. As of now, it remains unclear if and how these observed effects can be exploited practically; however, the results corroborate the advantageous use of whole cell catalysts. While further research will hopefully also allow to understand these results mechanistically, this work clearly emphasizes the shortcomings of comparisons across independent studies. For these reasons, we refrain in the following from comparing absolute values and focus on relative improvements when kinetic stability data—such as half-lives—are concerned. A parameter to reliably measure in a reproducible manner, however, is thermodynamic stability, which is indicated by a T_m, defined as the midpoint of a melt curve reflecting the unfolding of a protein ensemble.[246,247] This parameter is convenient in initial screens, as it requires little amount of sample, is nonlaborious and quick, and can easily be employed in a semihigh-throughput manner. For BVMOs, a method exploiting flavin fluorescence termed ThermoFAD allows T_m determination without the usually required addition of dyes.[246]

To improve the poor stability of AcCHMO, several groups have employed enzyme engineering—a task that has been complicated by the absence of a crystal structure. The first report of a more stable AcCHMO mutant targeted the oxidative stability of the enzyme, rationalizing that the hydrogen peroxide side product could inactivate the enzyme through oxidation of sulfur-containing residues.[248] By mutating all cysteines and methionines to amino acids found in homologous BVMOs, several positions were identified to increase substrate conversions in the presence of hydrogen peroxide and at elevated temperatures. The best variants of the subsequently generated combinatorial mutants showed a strongly increased hydrogen peroxide tolerance and a 7 °C upshift of the temperature at which 50% of activity remained. With the aim of increasing the thermal stability of AcCHMO, two parallel studies later created a homology model of the enzyme and used computational prediction to design stabilizing disulfide bridges. The first study reported an increase in T_m of 6 °C and a >10-fold increase in half-life at 37 °C for the best mutant, which interestingly was a disulfide bridge that spans only a single residue.[182] Combining several disulfide bridges led to strongly reduced expression levels, however. The second study tested four disulfide bridge designs and found an increase in T_m of 5 °C for the best variant.[183] Upon finding that the stabilization occurs even though the disulfide bridge does not form in solution, the individual mutations were tested, and the effect thus traced to a single threonine to cysteine exchange. This variant had a 6 °C higher T_m, and a ∼ 15-fold increase in half-life. Stabilization upon cysteine introduction is a surprising result, seemingly in contradiction to the earlier study that aimed to remove sulfur-containing residues. Although no clear explanation exists, the oxidation by hydrogen peroxide in this particular area of the protein may not negatively affect protein stability and act as a scavenger of reactive oxygen species. Recently, an effort was made to combine AcCHMO's most promising stabilizing mutations by adding the single residue-spanning disulfide bridge to the two mutants with highest oxidative stability.[209] Although no T_m was reported, the resulting variants were tested for their efficiency in ε-caprolactone production in a converging cascade. Surprisingly, the combinatorial mutant performed inferior to wild-type AcCHMO. Even after a design of experiments (DoE) to optimize the process toward optimal reaction conditions for the best-performing mutant, no more than 21 mM of ε-caprolactone was obtained. Although autohydrolysis of the lactone contributed to decreased yields, the mutant was apparently unable to outperform wild-type AcCHMO, which the same group previously optimized for the same reaction using a biphasic system.[249] Acknowledging the difficulties in engineering AcCHMO without a crystal structure, one recent study used as an alternative scaffold CHMO from *Rhodococcus* sp. HI-31, which is similar to AcCHMO with respect to both activity and stability.[250] In this work, a previously developed computational approach called FRESCO was used to predict stabilizing point mutations. After identifying several stabilizing hits on single mutant level, a combinatorial mutant with eight amino acid substitutions and a T_m of 49 °C was obtained, which amounts to an increase of 13 °C over wild type. Although the mutant displayed a slightly reduced maximal activity, it still had an approximately 2.5 fold higher k_{cat} for cyclohexanone than the naturally more thermostable TmCHMO. Currently, the RhCHMO 8-fold mutant and TmCHMO appear to be the most promising biocatalysts for applications targeting cyclohexanone or its derivatives. However, a thorough comparison of all available variants using a standardized assay and optimized reaction conditions would be desirable.

As none of these enzymes reach the stability levels of PAMO, which has a T_m of 61 °C[238] and does not lose activity for several days when stored at room temperature,[169] alternative strategies used PAMO as the engineering scaffold. Where the long-studied AcCHMO's catalytic properties were often found to be excellent, PAMO mostly proved to be a relatively poor catalyst for synthetically interesting reactions. The biggest weaknesses were the limitation of the substrate scope to small aromatic ketones and PAMO's inactivity on cyclohexanone, which prevent an application in biotechnological nylon production.[169] However, engineering of PAMO could finally be based on rational considerations because the enzyme was crystallized right after its discovery and this represented the first structure of a BVMO (Figure 1A).[43]

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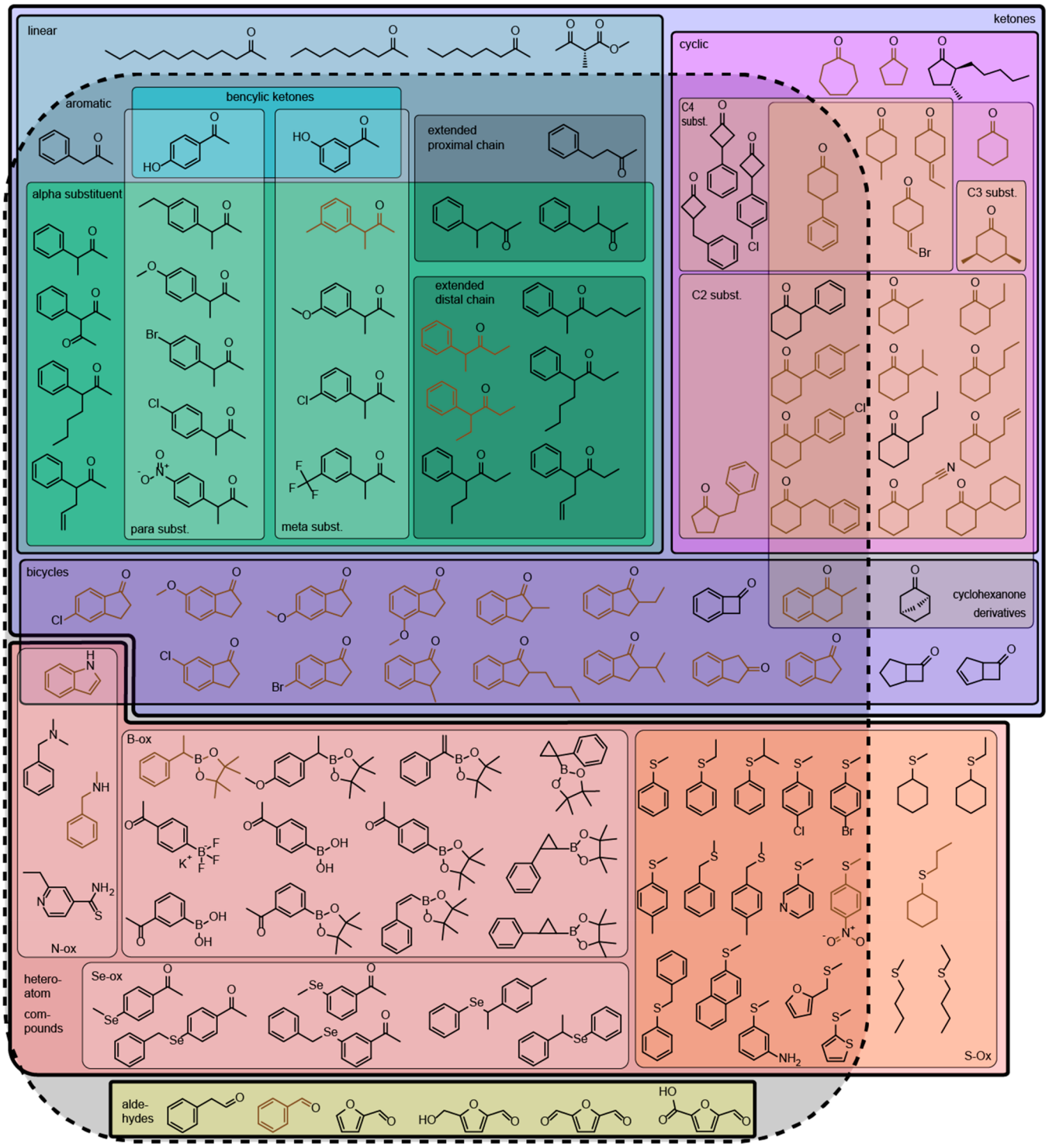
Scheme 5. Substrates Reported for PAMO and Its Mutants[a]



[a]Substrates are framed to indicate (overlapping) categories. Aromatic compounds are highlighted by a dashed line. Substrates for which activity was only reported with mutants are shown with brown carbons.

In six separate studies, the group of Reetz aimed at engineering PAMO toward activity with cyclohexanone and its derivatives (Scheme 5). Upon noticing that PAMO differed from CHMOs by a two-residue insertion in an active-site loop, this so-called "bulge" was deleted in the first PAMO engineering study[251] and subjected to random mutagenesis in the second (Figure 1B).[252] Although it was shown that either deletion or mutation increased PAMO's activity on cyclic ketones, the generated mutants were still limited to substrates containing the phenyl moiety. When the randomized region was then expanded to include additionally a subset of active-site residues, several multiple mutants with activity on 4-(bromomethylidene)-cyclohexanone emerged.[253] However, in a library targeting the other subset of active-site residues in addition to the bulge, the only mutations that emerged were single exchanges on the bulge.[254] Therefore, the authors decided to change strategy and

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targeted two conserved proline residues in the vicinity of, but not directly shaping the active site. Substitution of the proline directly adjacent to the bulge turned out to strongly increase enzymatic activity with a range of 2-substituted cyclohexanone derivatives that did not need to contain the phenyl group.[254] Concluding that successful proline mutagenesis may act by influencing conformational changes involved in catalysis, the authors expanded their investigation toward a proline found behind a loop they assumed to be critical for domain interaction.[255] Mutagenesis of this residue and a neighboring glutamate again increased the activity with cyclohexanone derivatives harboring substituents on C2 or C4. Interestingly, a strong cooperative effect of the mutations was observed: only a double mutant (Q93N/P94D) accepted these substrates, while none of the single mutants did. With these residues being far away from the active site, the authors suggested that they induced an allosteric effect that enables domain movements favoring the catalytic activity. In their most recent study, the authors eventually combined the previous hotspots and randomized the bulge residues, while fixing the mutations of the two proline residues and one neighbor.[256] This final mutant that contained two additional substitutions on the bulge showed for the first time an activity with cyclohexanone, although the low rate (k_{cat} = 0.3 s^{-1}) only allowed the conversion of 2 mM.

Most recently, a different group achieved conversions of 10 mM of cyclohexanone by combining the mutation of the conserved proline with a mutation of the active-site isoleucine identified as a hot spot by the Fraaije group.[257] This residue emerged in a study in which they designed mutations on the basis of a structural comparisons with a model of another promiscuous BVMO, cyclopentanone monooxygenase from *Comamonas* sp. NCIMB 9872 (CPMO). Using site-directed mutagenesis, 15 PAMO residues were mutated in order to map out crucial residues in the active site.[258] In another report they identified an active-site methionine mutant (M446G) that improved the activity with aromatic compounds and increased the heteroatom oxidation activity.[102] Interestingly, this mutant was able to produce indigo by converting indole through an apparent N-oxidation mechanism. The Gotor group and others subsequently characterized the substrate scope of this variant extensively and demonstrated its usefulness as a catalyst for the often enantioselective conversion of various compounds, most notably aromatic ketones[259−263] and heteroatoms.[115,131−133,264,265] The crystal structure of the mutant was also solved, and the same study also reported the first crystal structures of PAMO with a substrate analogue in the active site, which allowed to further narrow down the residues important for substrate binding.[59] With this combined insight, 11 residues were then chosen for simultaneous randomization.[193] A screen for enzymatic activity on cyclopentanone and cyclohexanone was conducted for 1500 clones, which still represented only a fraction of the statistically possible mutant combinations, however. A single clone containing four substitutions was identified in this screen that had activity on cyclopentanone. One mutation targeted a bulge-adjacent residue that also emerged in the Reetz libraries,[256] and three mutations occurred in residues located slightly further up the tunnel leading toward the active site. The biochemical characterization of this mutant showed that it had a strongly expanded substrate scope and accepted various aliphatic ketones. A theoretical study then used MD simulations to rationalize the selectivity alterations and found that the tunnel mutations cooperatively led to active-site rearrangements that stabilized the binding of the aliphatic substrate.[266] When a recent study also found indications for an important role of the substrate tunnel by identifying in it a stable binding site for ligands, a drastic engineering attempt was conducted: to establish whether the tunnel might be the true determinant of substrate specificity, two mutants were created, which switched the entire tunnel (25 mutations) or the tunnel and the active site (38 mutations) for the residues found in a CHMO.[56] This attempt turned out to be unsuccessful, however—although the mutants could be produced and bound FAD, barely any catalytic activity with a range of substrates remained. A similarly drastic approach was conducted in a study employing subdomain shuffling, which resulted in the creation of enzyme chimera.[238] Exchange of PAMO's C-terminus, which harbors the active site bulge and a large, mobile loop suspected to influence catalysis[50] resulted in chimeric variants with altered, but mostly PAMO-like activity. Collectively, these studies have generated hundreds of mutants with altered substrate and selectivity profiles. The mutants and their explored substrates were collected in an extensive table (see Supporting Information). As most studies compared mutant activities to the wild type, the substrate scope of native PAMO is now well explored. We found close to 90 compounds that were reported to be substrates of PAMO and a similar number reported for the M466G mutant (Scheme 5). Although PAMO clearly prefers aromatic compounds, it is rather substrate promiscuous among those. Considering that benzene is the single most common functional group in pharmaceuticals,[267] this may also be seen as an asset.

Domain movement may play a more important part in substrate acceptance than anticipated so far—in AcCHMO, mutations in the hinge region connecting the FAD and NADP domain had a profound effect on catalytic activities.[268] Since the enzyme is already naturally promiscuous—with the number of reported substrates in the hundreds[201,269,270]—only few other studies aimed at altering its substrate scope. With CHMO's main limit being substrate size, these efforts were often with a commercial interest, aiming to generate highly evolved variants optimized for a specific bulky target—commonly pharmaceuticals. A prominent example was the development of mutants with high sulfoxidation activity on the precursor of esomeprazole.[42,271] In another report, novel activities on exo tricyclic ketones was discovered for mutants originally evolved to switch product specificity.[272] Conversion of steroids is also of potential pharmaceutical interest, but engineering of CHMO is less appealing, as there are several BVMOs available that naturally accept steroids.[47,210,273] In these cases, the main challenge for biotechnological application is the poor water solubility of the substrates, highlighting the need for robust variants with good thermo- and solvent stability.

A number of stereoselectivity engineering examples are found in the literature, and dedicated reviews exist.[274,275] Many beneficial mutations have been identified by random mutagenesis, and a successful technique to reduce screening effort was the creation of focused libraries that target residues close to the active site.[16,276] A popular class of BVMO substrates are substituted cyclohexanones. For example, building on the previously discovered PAMO mutants with activity on such compounds, the Reetz group used iterative saturation mutagenesis to develop mutants for stereoselective lactone production.[253] Using a small set of appropriate substrates, they could introduce high regio-, enantio- as well as diastereoselectivity for the two best variants. Using a similar approach with a thermostable CHMO as the catalyst, a

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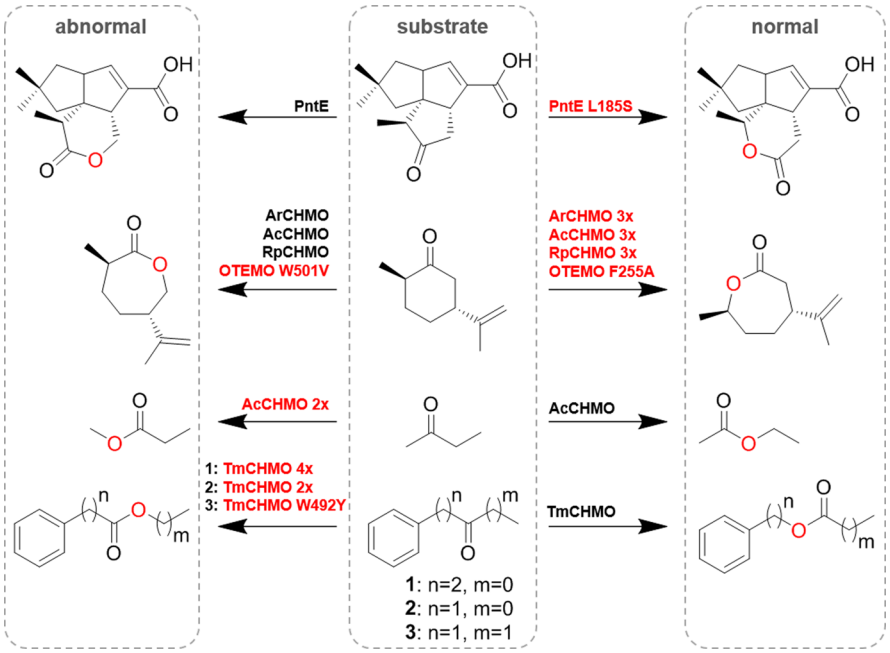
Scheme 6. Engineering of BVMOs To Change Regioselectivity[a]



[a]Mutants are highlighted in red.

stereoselectivity switch from *S* to *R* was achieved with 4-methylcyclohexanone.[277] In AcCHMO, a single mutation from phenylalanine to serine was found to be enough to completely reverse enantioselectivity of 4-hydroxycyclohexanone.[278] The result was rationalized in a theoretical study, which proposed that the reaction outcome is governed by the substrate's conformation and that the serine−substrate hydrogen bond allows an *R* product-favoring equatorial arrangement.[87] The same mutant also showed a drastic increase in *R*-selectivity for the sulfoxidation of thioether, but other mutations were found to lead to catalysts with higher activity, and some mutants were also found with reversed enantioselectivity.[125] In PAMO, directed evolution induced a selectivity switch from 90% *S* to 90% *R* in a variant with four mutations.[279] Interestingly, the four respective amino acid exchanges barely had an influence on selectivity as single mutations, indicating a high degree of mutational synergy.

As oxygen insertion can occur on either side of the carbonyl group, the Baeyer−Villiger reaction can also afford two regioisomeric products (Scheme 2). Regio- and stereoisomerism is often intimately connected in Baeyer−Villiger reactions e.g., with prochiral 4-substituted cyclohexanones where no stereocontrol can distinguish substrate enantiomers, but the side of oxygen insertion (i.e., regioselectivity) still determines the product enantiomers.[275] As specified before, the regioselective outcome is dictated by various effects, which lead to a predictable bond migration in chemical transformations, while often resulting in the noncanonical products by enzyme catalysis (Figure 2). In this case, the resulting ester has been referred to as "abnormal", while canonical bond migration affords the "normal" ester. In the case of cyclic substituted ketones, it was suggested to avoid ambiguity by using the terms "distal" and "proximal" lactones instead.[280,281] Although originally proposed for cyclic ketones with a substituent on the α carbon,[280] it has become more common for ketones with substituents further from the carbonyl.[282] Another interesting point is the often observed regiodivergent conversion of chiral racemic ketones, where the regioselectivity of the reaction differs for each enantiomer. This effect has frequently been observed to yield both regioisomers in a reaction, where each is produced enantiomerically pure or enriched. This behavior can be assessed with substrates such as *rac*-bicyclo[3.2.0]hept-2-en-6-one. This bicyclic ketone has become a model substrate[211] because it was first used to demonstrate BVMO-mediated asymmetric synthesis;[283] and an industrial, BVMO-catalyzed process has been established.[284] Being of a rather unpredictable outcome, the specificity of a large number of BVMO variants with countless substrates have been collected in extensive reviews.[269,270,282,285] Before the availability of crystal structures, the site-specificity of BVMOs has been the subject of controversial debate, and it has been tried to use in mapping the active site of AcCHMO based on the selectivity with various structurally restrained substrates.[286,287] While the models were still refined after the PAMO structure was available,[288] the subsequent RhCHMO structures and technological development led to an increased use of computational methods. Considering the complexity of the reaction mechanism and the partial uncertainty concerning conformational changes, however, it is maybe no surprise that most protein engineering studies still largely rely on random or semirandom libraries and use computational tools analytically rather than predictively.

A case of more targeted engineering was a BVMO involved in the *Streptomyces arenae* biosynthesis of pentalenolactone D (Scheme 6).[289] While this antibiotic features an abnormal lactone moiety, it was found that a homologous strain produces the metabolite as the normal isomer.[170] A few differing residues were identified by sequence alignment of the responsible homologous BVMOs and a single amino acid exchange in PntE was sufficient to completely inverse the selectivity of the abnormal lactone-forming enzyme. The opposite mutation in the natively normal lactone-forming enzyme did not cause abnormal product formation, however, and the enzyme was moreover largely expressed in inclusion bodies. The unique ability to deliberately produce abnormal esters is one of the synthetically most interesting features of BVMOs. Yet, also most

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other regioselectivity engineering studies reported a switch of selectivity toward the normal ester. The prevalence of this kind of "demolishing" of regioselectivity is likely because abnormal migration needs to be strictly enforced by the active site through steric control, while normal migration occurs also in the absence of a strict restraint via electronic control. Besides following the logic that the flavin intermediate underlies the same chemical principles as any other reactant, this notion was also substantiated by combined QM/MM studies[55,143] and an unusual experimental approach: upon cumulatively removing all active-site residues involved in substrate-binding, it was observed that the regioselectivities of a CHMO approached the ratios obtained with chemical catalysts.[56] A popular target molecule has been the terpene *trans*-dihydrocarvone, a 2- and 5-substituted cyclohexanone derivative with two chiral centers that many BVMOs can convert with high selectivity (Scheme 6).[290] In a traditional alanine-scanning mutagenesis experiment for the active-site residues of a CHMO, the Bornscheuer group already noted that exchange of one particular residue led to a switch in regioselectivity from fully abnormal to mostly normal lactone.[291] After two additional alanine mutations were introduced based on the alanine-scanning result and a docking experiment, the resulting mutant produced exclusively the normal lactone. Interestingly, all targeted residues were phenylalanines. The authors successfully transferred the mutations to AcCHMO, where the effect prevailed. When they later probed the mutations in OTEMO, they discovered that a single substitution—corresponding to the mutation showing the strongest effect in CHMO—was sufficient to induce exclusive production of the normal lactone in OTEMO.[292] The results were subsequently rationalized in a study from Scrutton and co-workers, who introduced the same mutations in another CHMO, solved the crystal structure of the mutant, and performed computational analyses.[55] As expected from three phenylalanine to alanine mutations, it appears that the mutations removed steric restraints exhibited by the wild type, thus inducing the reaction fate to be determined by the lower energy barrier associated with normal lactone production. Although the mutations proved to be mostly transferable among enzyme variants, they did not exhibit the same effect on other substrates. For example, the triple alanine mutant produced only slightly more than the 50% of normal lactone seen in conversions of (−)-bicyclo[3.2.0]hept-2-en-6-one with OTEMO, while a double valine substitutions achieved 95% normal lactone.[292] Surprisingly, the authors could not reproduce the result when using purified enzymes instead of whole cells, although they could partially restore selectivity by adding FAD to the purification buffer. This effect was, however, not observed for another mutant, already known earlier to influence selectivity,[53] in which a conserved tryptophan to alanine mutation caused 95% abnormal lactone production. Mutagenesis-induced activity increase toward abnormal ester—the more interesting, though, more challenging task—is commonly only observed sporadically. For example, the PAMO M446G mutant was found to convert 1-indanone and its derivatives to the abnormal lactone.[259,262] Targeted engineering of abnormal product formation has been attempted in several studies for 2-butanone (Scheme 6). The reaction is of synthetic interest, as the abnormal product, methyl propanoate, can be converted to methyl methacrylate, an acrylic plastic produced industrially on megaton scale annually.[293] An initial screen of several BVMOs showed moderate activities with most BVMOs, and the best enzyme, AcCHMO, produced approximately 25% abnormal product.[294] A small library based on structure-inspired rational design was then tested for improvement, and a double mutation identified, which increased the yield and produced 43% of the abnormal product.[295] The fact that a full switch was not achieved reflects the apparent difficulty in engineering a preferred migration of the least favored substituent, the methyl group. Recently, this was nevertheless achieved with even more demanding substrates—aromatic ketones in which the energetically least-favored methyl group competes with phenyl substituents (Scheme 6)—by screening larger libraries and several rounds of directed evolution.[143]

BIOTECHNOLOGICAL APPLICATIONS

Biotechnological Application: Obstacles. The application of BVMOs is partially characterized as troublesome because of a number of important limiting factors, including enzyme expression,[219] enzyme stability,[248] NADPH-dependence,[296,297] oxygen-dependence,[180] and substrate and product inhibition.[208] However, depending on the specific BVMO, there will be specific obstacles; for example, some BVMOs have good expression, yet poor stability, or vice versa. In this subsection, we will discuss each of these limitations and refer to studies that have addressed them. First of all, the application of BVMOs can be carried out in four different forms: with isolated enzymes, with immobilized enzymes, with crude/cell-free extract, or with whole cells. Most commonly, application-oriented reactions applied whole cells, as they provide a number of advantages: (1) improved stability of the enzymes due to the cellular environment,[20] (2) no addition of NADP(H) is needed, (3) coexpression of other enzymes can facilitate cofactor recycling or cascade reactions, (4) no cell lysis and enzyme purification steps are needed, and (5) it allows for continual expression of the enzyme(s). However, there are also some disadvantages with whole cells, such as (1) mass balance issues and product removal,[298] (2) problematic oxygen supply to the cells,[180] (3) plasmid stability with requirement of antibiotic,[299,300] and (4) limited transport of substrates/products in and out of the cell.[301] In addition, a study on a cascade reaction in vivo, where a kinetic model was used to analyze performance, revealed that cofactor concentrations in the cell were limiting the reaction rate.[179] Possibly, this challenge could be addressed through metabolic engineering or the use of a different host. Still, each of the ways to apply BVMOs has trade-offs, and it will be case-specific whether one is more suitable than the other. A recent minireview addresses some of these aspects that are relevant for the development of a biocatalytic (industrial) process.[302]

Industrial Demand, TTN, and Stability. Most studies on BVMOs describe reactions on small lab-scale. Yet, to meet the demands of an industrial process, the limiting factors presented above need to be addressed. Specifically, to produce low-priced compounds, such as building blocks for polymers, a ratio of 2000−10000 g of product/g of (immobilized) enzyme (also referred to as "biocatalyst loading") should be met in order to be an economically viable process.[20] To illustrate, assuming a 100 g mol^{-1} product and a 50 kDa enzyme, 20−100 mol of product/g of enzyme, the demanded ratio translates to $1 \times 10^6 - 5 \times 10^6$ total turnovers (TTN) per enzyme. Because of these numbers, many BVMOs are still excluded from industrial application, unless the target product is of high value, as is the case for pharmaceuticals, or if effort is invested to improve the biocatalyst and the process. In particular, improvement of the stability of the biocatalyst is needed in many cases, as is discussed in the subsection of Enzyme Engineering of this Review.

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Table 5. Preparative-Scale Reactions with BVMOs

product	product concentration (g/L) + isolated yield	enzyme	biocatalyst yield ($g_{product}/g_{enzyme}$)	TTN[b]	ref
esomeprazole	50 (~151 mM) 87% yield (28.7 g)	AcCHMO (multiple mutant)	50	8841	271
bicyclo[3.2.0]hept-2-en-6-one lactone	4.5 (~41 mM) 55% yield (0.49 kg)	AcCHMO	3[a]	n.a. (wc)	284
(*Z*)-11-(heptanoyl-oxy)undec-9-enoic acid	41 (132 mM) 68% yield (75 g)	*Pseudomonas putida* BVMO (E6-BVMO C302L)	1.6[a]	n.a. (wc)	218
3,3,5-trimethyl-caprolactone	24.4 (~156 mM) 76% yield (1.9 kg)	TmCHMO	0.6[a]	n.a. (wc)	314
6-hydroxy-hexanoic acid	20 (~151 mM) 81% yield (8.1 g)	AcCHMO C376L/M400I/T415C/A463C	0.7[a]	n.a. (wc)	315
lactone of (2*R*, 5*R*, 6*R*)-6-methyldihydrocarvone	0.82 (4.5 mM) 90% yield (49 mg)	CHMO_Phi1	6.7	2250	295
precursor of Nylon-9	8 (70 mM) 70% yield (33 g)	CPDMO	2.3[a]	n.a. (wc)	316

[a] $g_{product}/g_{cdw}$ = gram product per gram cell dry weight, and $g_{product}/g_{cww}$ = gram product per gram cell wet weight. TTN = total turnover number, representing the amount of molecules converted per molecule of enzyme. [b] Total turnover number. wc = whole cells.

Moreover, an important issue for both the metric "total turnover number" (TTN) and "stability" is their inconsistent use in studies so far. Many studies refrain from determining the TTNs, and stability is often described in different ways, such as melting point and half-life, which makes it difficult to compare data. In addition, the stability of an enzyme in an industrial setting could be different compared with the lab setting. What could help the field of BVMO biocatalysis in general is if studies provided data for these characteristics, or at least a clear description of biocatalyst loading because this metric gives an impression of the efficiency and stability of the biocatalyst, whether it is whole cells or isolated enzymes.

NADPH Recycling. While expression and stability are more related to enzyme engineering, the efficient use of NADPH is primarily determined by the way the BVMO is applied. The dependence of BVMOs on NADPH is an important challenge, since the cofactor is expensive: around 1800$/5 g, compared to 280$/5 g for NADH.[303] Therefore, it is necessary to minimize the amount of NADPH that is used. One way to address this challenge is to devise a set of reactions that are in redox balance. This can be achieved by combining an oxidation reaction, in which the reduced cofactor is formed, with the BVMO reaction, in which the reduced cofactor is oxidized again.[304] A typical example of such a redox-neutral reaction is the combination of a BVMO with an enzyme that can catalyze the oxidation of a sacrificial substrate (e.g., glucose dehydrogenase).[296,297] Alternatively, whole cells can be used for internal cofactor regeneration,[305,306] as well as nonenzymatic ways.[307] Nicotinamide cofactor regeneration strategies have been extensively reviewed elsewhere.[297] Another type of a redox-balanced pair of reactions are cascade reactions, where the product of the first reaction is the substrate for the second reaction.[308] One advantage of cascade reactions is that the isolation of intermediate products is not needed, as can be the case in other synthesis routes. In general, there are quite well-studied solutions to the challenge of cofactor recycling, with examples given in the next subsection.

Oxygen Supply. Another degree of complexity of industrial application is oxygen supply. Because a stoichiometric amount of oxygen is needed for the typical BVMO reaction and aqueous solutions contain about 0.25 mM of oxygen at 25 °C, effective oxygenation of the reaction mixture is needed to convert higher concentrations of substrate. However, supplying pure oxygen directly can be dangerous because of the risk of explosion. Studies that describe the oxygen limitation for biocatalytic oxidations include a study on whole cells expressing AcCHMO to convert bicylco[3.2.0]hept-2-en-6-one.[180] The authors observed a limitation of oxygen at a particular cell density (>2 g_{cdw}/L), above which the reaction rate was decreased. In this regard, it seems that the use of whole cells can be a disadvantage compared with isolated enzymes because cells seem inefficient at taking up oxygen.[180] To improve cellular concentrations of oxygen and study the effects, BVMO reactions were tested in whole cells coexpressing a bacterial hemoglobin gene.[309] It was observed that the presence of the bacterial hemoglobin gave a 43% improvement compared with the control, conversion of cyclohexanone by CHMO.

To address the limitation of oxygen for larger scale reactions, one can employ devices that can effectively transfer oxygen to a reactor.[310,311] A recent study described a strategy to monitor the oxygen concentration of a reaction, providing accurate values, despite the presence of multiple phases (such as biphasic systems).[312] Although typically the subject of oxygen limitation is addressed at the process stage of a BVMO application, oxygen monitoring technologies can help identify oxygen limitation at an early stage, such that a solution can be prepared before scaling up.

Product and Substrate Inhibition. Because product inhibition appears to be a general issue for CHMOs, a few studies addressed this specific aspect. In one case, AcCHMO was subjected to mutagenesis to make variants which suffer less from product inhibition.[313] In this study, 4200 clones were screened, resulting in 6 hits, with the best mutant displaying a 2-fold higher resistance compared with wild-type CHMO at 600 mM ε-caprolactone. Engel et al. recently characterized a BVMO from *Aspergillus flavus*, which had no sign of inhibition from substrate or product using concentrations up to 100 mM and compared conversions with a few CHMO variants.[209] In other studies,[179,207] the authors sought to minimize inhibition by optimizing cascade reactions through computational simulations, combined with in vitro experiments. The computational simulations incorporate many variables, such as the kinetic properties of the enzymes and then predict the course of the reaction. In principle, the use of a cascade reaction with an alcohol dehydrogenase, starting from cyclohexanol, will keep the cyclohexanone concentrations low, thereby addressing some substrate inhibition. Nevertheless, it is a delicate task to optimize all parameters, such as the rate of substrate feeding, and reliable data of kinetic parameters is very important in order to make accurate predictions. When substrate and/or product inhibition

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are observed, the approach of kinetic modeling could be valuable to pinpoint bottlenecks, and help to choose the right solution. Alternatively, new enzyme variants or mutants could be identified, which address this important shortcoming.

Biotechnological Application: Scaled-Up Applications. Until now, only a few examples of industrial-scale applications of BVMOs are known (Table 5). One actual industrial process involves a CHMO variant for the enantioselective sulfoxidation of pyrmetazole, to produce esomeprazole.[271] Initially, the wild-type CHMO from *Acinetobacter* had barely any activity on the substrate. After 19 rounds of evolution, partially random and partially rational, the biocatalyst had several improved features: high activity (~140 000 fold improvement in productivity over wild-type), enantioselectivity (>99% ee), low percentage of overoxidation to sulfone, and high cofactor efficiency (less $NADP^+$ was needed). After process optimization, reactions at 50 g/L substrate could be performed, resulting in a yield of 87%, with 99.9% ee and 99% purity (based on HPLC). Although total turnover numbers are moderate (Table 5), they are acceptable for such a high-value product. The flipside of this example is that most research groups and companies do not have the means (in terms of equipment and/or financial) to perform 19 rounds of evolution and to do this kind of process optimization. Although studies are finding promising results, the academic route of biocatalyst development is comparatively slow, which partially explains the lack of industrial application of BVMOs.

Another BVMO-based application that has been studied to bring to industrial scale is the regioselective oxygenation of long-chain fatty acids into esters. These ester products can subsequently be hydrolyzed to obtain valuable medium-chain acid products, including ω-hydroxycarboxylic acids, α,ω-dicarboxylic acids, and ω-aminocarboxylic acids.[317] Interestingly, activity on such substrates is the primary function of many BVMOs in nature, as they are often coexpressed with esterases that catalyze subsequent hydrolysis of the esters. Several BVMOs have been identified that can act on long-chain fatty acids, in particular BVMOs from *Pseudomonas* species,[217,318,319] such as PaBVMO from *Pseudomonas auruginosa*, and PpKT2440-BVMO from *Pseudomonas putida*.[217] The PaBVMO was recently characterized and is able to produce the abnormal ester products from long-chain aliphatic keto acids (C16−C20), which—after hydrolysis—yielded α,ω-dicarboxylic acids that are otherwise difficult to produce.[319] The PpKT2440-BVMO could be applied in combination with an ADH from *Micrococcus luteus* to convert ricinoleic acid to (*Z*)-11-(heptanoyloxy)undec-9-enoic acid. However, this BVMO was difficult to express in *E. coli*, and to improve expression, several strategies were explored, including the use of chaperones,[181,320] enzyme fusion,[320,321] polyionic fusion tag engineering, and the use of a constitutive promotor.[219] Through the polyionic tag and constitutive promotor, the reaction could reach a product concentration of 21.9 g/L on a 70 L scale. To further improve the whole cell application for the conversion of ricinoleic acid, and other long chain fatty acids, more strategies were applied: coexpression with the fatty acid transporter FadL,[320,322,323] stability engineering of the BVMO,[218,324] glucose feeding for improved metabolic stability,[218] and use of a stable plasmid system.[324] Through the stability engineering and glucose feeding, a product concentration of 41 g/L (132 mM) within 8 h could be achieved (on 3 L scale). Recently, an overview of enzymatic fatty-acid transformations was published, including many cases that involve BVMOs.[325] These studies illustrate the work that is needed to bring lab-scale biocatalysis of BVMOs to an industrial bioprocess. Key obstacles in the case of PpKT2440-BVMO were expression and stability.

Because some BVMOs are able to produce lactones from cyclic ketones, which can be used to make various polyester materials, studies have looked into scaling up the production of lactones with BVMOs. The Mihovilovic group could demonstrate the production of a Nylon-9 monomer on 40 g scale, employing CPDMO in whole cells and carefully addressing substrate and product inhibition through substrate feeding and product removal (Table 5).[316] An incomplete conversion of around 75% could be reached, which was attributed to the particular reactor at hand, which could not ensure proper mixing of the ketone substrate. The authors recommend future studies to monitor the reaction through off-gas measurements, similarly to Meissner et al.[312] To explore the synthesis of branched polyesters from biobased sources, Delgove et al. investigated the biotransformation of a set of seven substituted cyclic ketones with three self-sufficient BVMO fusions.[326] The abnormal lactone products, which can be formed by some BVMOs, could represent novel building blocks for polyester synthesis. Conversion was demonstrated for four of the cyclic ketones, two of which resulted in mixtures of normal and abnormal product. In subsequent work, the authors upscaled the transformation of 3,3,5-trimethylcyclohexanone. TmCHMO was used as biocatalyst because of its stability and solvent tolerance and was paired with PTDH as a fusion for cofactor recycling.[327] Crucially, some strategies were used to overcome substrate inhibition and product solubility. By employing slow substrate feeding, methanol as cosolvent, and a biphasic system with toluene as second phase to sequester the product, a respectable space-time yield (STY) of 1.2 g L^{-1} h^{-1} could be reached. In a similar study, TmCHMO was used in combination with a separate GDH as recycling enzyme, and in a setup with continuous substrate feeding, a STY of 1.35 g L^{-1} h^{-1} was reached.[328] One approach to greatly enhance the total turnover number of a biocatalyst is through immobilization and reuse of the immobilized biocatalyst. The same target reaction was also studied with immobilized TmCHMO.[329] GDH was either coimmobilized or individually immobilized and added separately to the reactions. The coimmobilized enzymes could be reused while maintaining full activity for five rounds and beyond that gradually decreased, obtaining 60% conversion after 14 reuses. Immobilization of enzymes for this conversion was recently optimized, by applying a different GDH, and testing different supports. Compared with the soluble TmCHMO and GDH, the immobilized forms had a 3.6-fold and 1.9-fold improvement, respectively, in terms of the biocatalyst yield (37.3 g g^{-1} for TmCHMO and 474.2 g g^{-1} for GDH).[330] To investigate the relevance of these biocatalytic studies on 3,3,5-trimethylcyclohexanone conversion in terms of applicability and environmental impact, a life-cycle analysis (LCA) was done.[314] The biocatalytic process[327] was compared with a chemical synthesis route, and no clear difference in climate change impact was found between the two routes. However, the environmental impact of the biocatalytic process would be lower compared with the chemical route when solvents and enzyme are recycled. The study details what factors to consider when developing a biocatalytic process, and the LCA approach can provide useful insight into the feasibility of a potential process.[314] A very recent study looked to scale up the reaction on 3,3,5-trimethyl cyclohexanone, with TmCHMO and GDH. First, the authors tested four different formulations: cell-free extract, whole cells,

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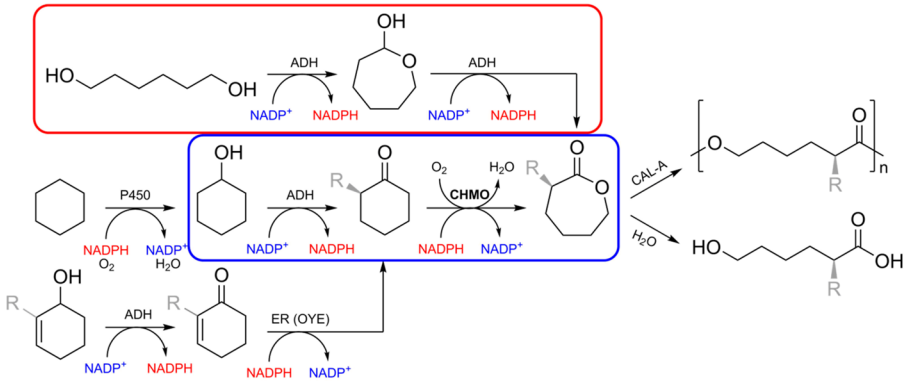
Scheme 7. Overview of Biocatalyst Combinations for Cascades Involving Cyclohexanol and CHMO[a]



[a]Blue box: redox-neutral cascade from cyclohexanol to ε-caprolactone. Red box: ADH conversion of 1,6-hexanediol, which can be combined with cyclohexanone conversion by CHMO to recycle NADPH.[249] A cascade starting from cyclohexane involving a P450 monooxygenase was described.[342] Unsaturated cyclic alcohols or unsaturated cyclic ketones can be used with ene-reductase (ER) cascades, to make chiral lactones.[343,344].

fermentation broth, and sonicated fermentation broth. For TmCHMO, the untreated fermentation broth was chosen for performance, and least amount of costs, and GDH was added separately. On a 100 L scale, the 2.58 kg obtained material contained 84.5% product, and the final yield was 76%.[331] Although the biocatalyst yield (gram product per gram enzyme/cells) seems much lower compared with the same reaction with immobilized enzyme,[330] it is actually difficult to compare these values, since a lot of costs go into the preparation and immobilization of the enzymes, in contrast to the fermentation broth used in this study.[331] This demonstration is a great example of how to reach large scale production with a BVMO reaction by addressing important challenges, in particular the oxygen limitation through careful supply of pure oxygen. Despite the success, it would be interesting to see whether the low levels of cofactor in the cells affected this particular reaction, as was described by Milker et al.[179]

The low number of preparative-scale applications (Table 5) can be explained by considering the obstacles that arise from upscaling. Oxygen limitation and substrate inhibition have been successfully addressed in recent demonstrations,[316,331] although one could argue that oxygen supply remains suboptimal with respect to using whole cells.[180] On the other hand, obstacles such as enzyme stability and product inhibition are not easy to solve in any general way, since these will be case specific due to the use of different BVMOs and different products. A recent review on preparative-scale biotransformations with redox enzymes states that a better characterization of BVMOs, together with addressing oxygen mass transfer, will bring more applications in the future.[332] Yet, we want to emphasize that the characterization of BVMOs, or development of BVMO variants, that meet the requirements of an industrial application (high expression, high stability, low inhibition, high enantioselectivity) is a major challenge. From the examples (Table 5), we can conclude that scaling up a BVMO reaction typically demands (1) resources, such as time to engineer the biocatalyst[218,271] and the process, and (2) facilities, such as proper reactors with equipment to control oxygen levels.[316,331] Successful industrial implementation of BVMO-processes will rely on companies that can bring together expertise in enzyme and process development.

Biotechnological Application: Examples of Cascade Strategies and Novel BVMO Applications. Over the past decades, some progress was made in optimizing large-scale reactions, employing strategies such as biphasic systems,[333] whole cell conversions,[305] and enzyme immobilization.[329,334,335] Reviews focusing on biocatalysis with BVMOs from prior years are referred to for a broader overview.[201,336−338] Alongside these developments, several groups have explored different reactions and combinations of reactions with BVMOs, of which we present an overview, focused on studies from recent years. In particular, these combinations of reactions include cascades, as well as chemoenzymatic routes.

To facilitate cofactor recycling, an elegant strategy is to use a cascade reaction. For BVMOs, a frequently researched example is the cascade reaction with CHMO and an alcohol dehydrogenase (ADH), starting from cyclohexanol (Scheme 7, blue box). The alcohol oxidation generates NADPH and cyclohexanone, which is then oxidized by CHMO to ε-caprolactone. Several groups investigated and developed this cascade reaction.[208,315,339] Initially, problems were encountered concerning substrate and product inhibition. Higher levels of conversions could be achieved by keeping the substrate concentration low, through slow feeding, and removal of the lactone product by a subsequent polymerization/hydrolysis using a lipase such as CAL-A (Scheme 7).[340] This biocatalytic route was recently applied in whole cells that coexpress CHMO and ADH on a 0.5 L scale, feeding of cyclohexanol, and addition of a lipase for hydrolysis of caprolactone to 6-hydroxyhexanoic acid (Table 5).[341] After optimization, the process at 0.5 L scale could reach a product titer of 20 g L^{-1}, with an isolated yield of 81% of 6-hydroxyhexanoic acid.

To address the cofactor balance, a different kind of cascade reaction was developed by Hollmann and Kara.[249] With the production of lactones in mind, an alcohol oxidation reaction of a linear diol was run in parallel in one pot with a Baeyer−Villiger reaction on a cyclic ketone catalyzed by CHMO (Scheme 7, red box).[249] As alcohol oxidation by an alcohol dehydrogenase (ADH) depends on $NAD(P)^+$ and produces NAD(P)H, combining this reaction with a BVMO or FMO reaction brings a redox balance. When one alcohol group of a linear diol becomes oxidized, the molecule undergoes cyclization to the hemiacetal or lactol. This lactol can be oxidized again to form a lactone (Scheme 7, red box). However, since the ADH generates two molecules of NAD(P)H in the conversion of one diol to one lactone, the substrate concentrations should be 2:1 of FMO

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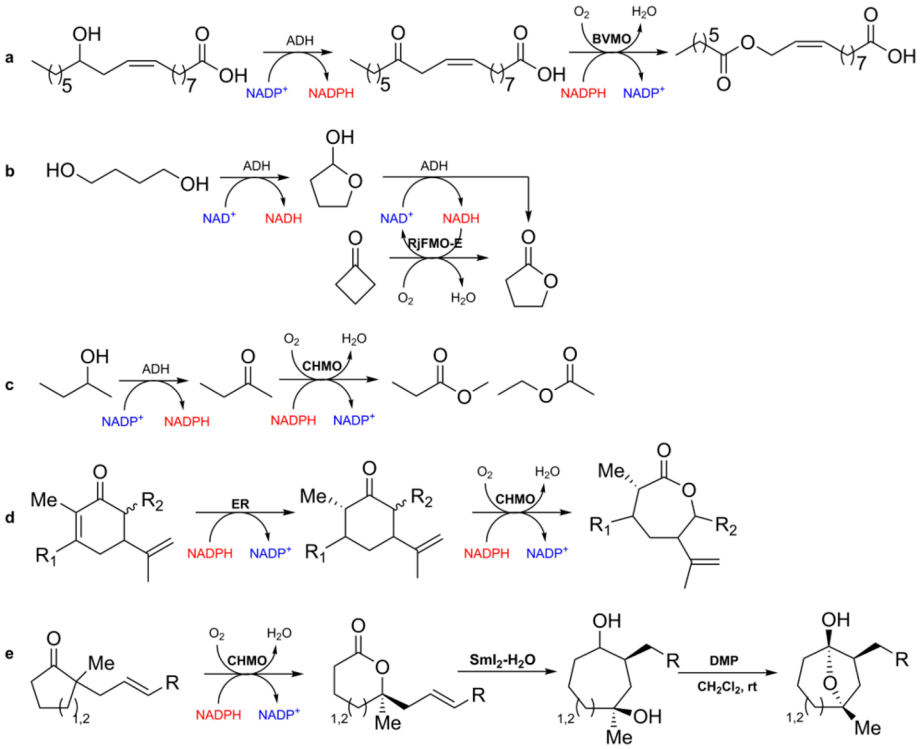
Scheme 8. Examples of Cascade Reactions Involving BVMOs[a]



[a](a) Conversion of hydroxylated long-chain fatty acids to produce esters.[181,218,219,320−323] The cascade could also start from an unsaturated precursor with a hydratase to make the hydroxyl group[320] or with a P450 to perform hydroxylation. (b) Convergent cascade analogous to the reaction displayed in Scheme 7, red box.[249] This particular cascade relies on NADH, through the use of RjFMO-E.[162] The same reaction was also used with fused enzymes in organic solvent.[348] (c) Cascade from 2-butanol to methyl propionate (the first product, abnormal), a precursor for methyl methacrylate.[82,83] (d) Various monoterpenoid ketones can be transformed to obtain chiral lactones, through ene-reduction by an ene-reductase (ER) or old-yellow enzyme (OYE) followed by lactonization by CHMO.[355−357] (e) A chemoenzymatic route that starts with Baeyer−Villiger oxidation, which is enantioselective (> 99% ee), and is followed by a SmI_2−H_2O mediated radical cyclization reaction.[358] The cyclization is completed by the third step, which is an alcohol oxidation by DMP (Dess−Martin periodinane) in dichloromethane. The two chemical steps maintained the chirality of the CHMO product, and the final products could be obtained with >99% ee.

substrate to ADH substrate. This approach was termed convergent cascade, since two different substrates converge to the same product; the lactone. The Kara group later made an analogous combination, to produce γ-butyrolactones using RjFMO-E (Scheme 8b).[162] An interesting aspect of that study is that the FMO that was used to perform the Baeyer−Villiger reactions could accept NADH, making it a more feasible process compared to an NADPH-dependent reaction, considering the higher cost of NADPH[303] and its inferior stability.[345]

A related strategy is to create a fusion of a cofactor recycling enzyme with a BVMO. This approach enables coexpression of both enzymes (as a fusion enzyme) and simplifies purification, whole cell conversions, and coimmobilization. Enzyme fusions with BVMOs have been reviewed recently[346] and thus will only briefly be discussed here. One recent study looked at fusions of three cofactor-regenerating enzymes with TmCHMO: glucose dehydrogenase (GDH), phosphite dehydrogenase (PTDH), and formate dehydrogenase (FDH).[347] These were compared in conversions and tested with various substrates and cosolvents, including a deep eutectic solvent (DES). One particularly efficient setup consisted of a natural DES (NADES) with glucose and the GDH-TmCHMO fusion, in which the NADES enables higher substrate loading, while also containing excess glucose to push NADPH recycling by GDH. Recently, following up on the convergent cascade (Scheme 8b),[162] fusions of the ADH and FMO were created to produce γ-butyrolactone in an unusual setup, using organic solvent.[348] Studies in the past have found that enzymes can actually be more stable and active in an organic solvent, though the use was limited to lipases and esterases.[349] However, the ADH-FMO reaction is more challenging as it relies on NADH, which is why the authors chose to fuse the two enzymes. Cell-free extract from cells expressing the enzyme fusion was lyophilized and subsequently added to organic media with 5% (v/v) water, to which the two substrates (diol and cyclic ketone) were added.[348] Although the yield was limited (27%), the fusion enzyme was able to perform the cascade reaction in this microaqueous media, and outperformed the combination of the separate enzymes. Moreover, no external NADH was added, which is appealing in terms of applications.

The approach of enzyme fusion is also very suitable for multienzyme cascade reactions. In some cases, the fusion outperforms the combination of separate enzymes, which is linked to an effect of the proximity of the enzymes called substrate channeling.[350−352] In 2013, Jeon et al. developed fusions of ADHs with BVMOs to convert hydroxy fatty acids into esters, in whole cells expressing the fusion enzyme (Scheme 8a).[321] The authors could demonstrate that the fused enzyme had a higher level of conversion for the cascade reaction. A similar pair of ADH with TmCHMO was fused to produce ε-caprolactone from cyclohexanol.[353] Although the fusion was more productive than the separate enzymes, substrate feeding

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and product removal through a lipase were needed to obtain full conversions, as was described previously (Scheme 7).[340] This fusion of an ADH with TmCHMO was also applied for a process to convert 2-butanol to methyl propionate (Scheme 8c), which is a precursor for the plastic feedstock methyl methacrylate.[354] Prior to that study, TmCHMO was engineered to give higher conversion and a higher ratio of the abnormal product.[295] Another study fused CHMO with an ene-reductase to enable cascades starting from an unsaturated cyclic ketone, or unsaturated cyclic alcohol by including an alcohol dehydrogenase as third enzyme, to make chiral lactones (Scheme 7 and Scheme 8d).[344]

Other groups have also explored the potential of applying BVMOs for the production of lactones, in this case from monoterpenoid ketones, and followed-up with the polymerization of the products. In one study, a novel CHMO was applied, after characterization, crystallization, and engineering to steer the regioselectivity.[55] The CHMO_Phi1 structure was then used to perform modeling and simulations to explain the change in regioselectivity of the mutant. For menthone and dihydrocarvone, biocatalytic conversion could be demonstrated as well as polymer synthesis. In another study, analogues of (*R*)-(−)-carvone were used as precursors for a reduction reaction with Old-Yellow Enzymes (OYEs), and subsequent Baeyer−Villiger oxidation by CHMO_Phi1 (Scheme 8d).[355] Reactions could be scaled up to 100 mg, demonstrating preparative biocatalytic synthesis of chiral caprolactones (Table 5). Dihydrocarvide was also produced through a whole cell conversion by incorporating a BVMO and OYE, alongside a limonene synthesis pathway.[356] This proof-of-principle study demonstrated the production of a renewable bioplastic monomer starting from glucose, through precise expression of the required enzymes. An important aspect of the conversion of monoterpenoid ketones by BVMOs is the chirality of the substrate and/or product. This was demonstrated in a study which used combinations of enoate reductases and various BVMOs in cascade reactions to convert (+)- and (−)-carvone to six different carvo-lactone stereoisomers (Scheme 8d).[357] In a study by Stamm et al., a specific polyester with a ring was targeted, and using retrobiosynthesis, a chemoenzymatic route was devised to obtain the needed lactone from pinene.[359] For the BVMO step in that route, the biocatalyst was engineered in order to accept the substrate. Recently, an overview of (chemo)enzymatic routes for (lactone) monomer production was published, including several examples of BVMO reactions.[360]

Since the BVMO-catalyzed Baeyer−Villger oxidation is often very selective, it can be a synthetically useful way to access chiral precursors for various synthesis routes. One example is a chemoenzymatic approach where chiral lactones are produced with AcCHMO, which are then converted to cycloheptanols and cyclooctanols through a radical cyclization reaction involving SmI_2−H_2O (Scheme 8e).[358] The products from this approach contain structural components that are present in certain anticancer and antibacterial drugs. In another chemoenzymatic approach, Zhang et al. devised a cascade reaction starting with a photocatalyzed reaction, of which the product was subsequently converted with BVMOs, among other enzymes.[361] However, the two catalysts were incompatible, and higher conversions were obtained when the two steps were done separately. The challenges for applying such chemoenzymatic cascade reactions were discussed in a recent minireview.[362] Another chemoenzymatic route involving a BVMO to produce (R)-Taniguchi lactone was recently studied.[216] Two novel BVMOs were used, and the authors describe several other cyclic ketones that can be converted with these biocatalysts, with varying degrees of regio- and enantioselectivity. These recent examples show that BVMOs can be useful in particular synthesis routes and allude to a broader range of possible applications with these biocatalysts.

The development of strategies, like enzyme fusion, use of cosolvents, and cascade reactions have shown to be meaningful steps on a path toward biotechnological application. However, it is a path that still needs further exploration in order to meet the demands of an industrial process. The studies from the recent years show the variety of products that can be accessed through BVMOs. Given the limited turnover numbers that are reached in these studies, we conclude two things: (1) with the current state of BVMOs, any industrial application can only be economically feasible if the products are of high value (such as esomeprazole[271]), and/or through thorough optimization of the biocatalyst and process. (2) For the application of BVMOs for bulk chemicals (e.g., monomers) there are some examples,[316,331] though more work needs to be done with respect to biocatalyst loading (in other words: operational stability and activity). So far, some studies have moved in the direction of biotechnological application and have applied BVMOs for the synthesis of various compounds (Table 5). As BVMOs become more suited, reliable, and recognized for biocatalytic application, it is likely that more groups and companies will look to harness the utility that these biocatalysts can provide. Though, to realize scaled-up applications, joint efforts will be needed that bring together different expertise, ranging from enzyme engineering to process development, to effectively tackle the specific challenges.

CONCLUSIONS AND FUTURE DIRECTIONS

Biotechnology is at an exciting crossroad where ever more discoveries lead to the developments of applications in the various subdisciplines that have (e)merged. Biocatalysis is maturing to a serious alternative to classical chemical transformations and this hopefully can contribute to a greener industry and new products at the same time. Baeyer−Villiger monooxygenases are intriguing catalysts for a demanding reaction that allow unrivaled control of the reaction selectivity. Numerous variants have been described that feature activities suitable for countless reactions of synthetic value. Limitations, such as cofactor dependency, limited stability, and undesired specificities are clearly identified and active research is making progress in overcoming these. A suitable tool to that end is enzyme engineering, and directed evolution has been most effective in altering undesired enzyme properties. Computational design has a great potential, but needs to become more reliable—a task that will not least be facilitated by unravelling the last remaining mechanistic open questions of BVMO catalysis.

The most important open questions lie in the mechanism, and they may or may not all be connected to each other. The uncertainty about the kinetic step of BVMOs found to be rate-limiting needs to be resolved. If it turns indeed out to be a conformational change, its nature (side-chains, loops, cofactors, domains?) needs to be elucidated. In relation to that, uncertainties in the exact position of the substrate during catalysis must be clarified.[50] Only if these uncertainties are dispelled can computational analyses be taken fully seriously and used in a predictive way. This will hopefully largely reduce the

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workload currently required to engineer desired activities and allow the design of tailor-made mutants for specific substrates. Similarly, the puzzle of substrate promiscuity will then need to be connected to these insights. Possibly related is another mechanistic open question—the reduction of the flavin by NADPH. The incoherency of the stereochemistry of hydride transfer with the sliding mechanism is not just a curiosity,[50] it will also be essential in enabling the engineering of true dependency on the dephosphorylated cofactor.

Next, the issue of substrate and product inhibition has been largely unaddressed. The reason may be that so far, the low stability often has masked this limitation. However, with new homologues and engineered variants, this issue has become and will be the more important new bottleneck. Also here, an approach aiming to tackle the underlying cause, and not just the symptoms would be desirable. However, until cleverly designed experiments are able to establish the mechanism of inhibition and protein engineering can be applied to overcome it, another focus will lie in process design and engineering. Chemo-enzymatic systems employing (co)solvents and cascade reactions have already become popular and many more examples are expected to be developed in the future.

An extended knowledge will also be valuable for stability engineering, where seemingly distant mutations can sometimes abolish activity.[250,268] Although the stability of BVMOs has been tackled, it can be doubted that this is enough to reach a broad application. However, with so many thermo- and hyper-thermostable enzymes known from other enzyme families,[363] it seems fair to speculate that it is only a matter of time until a BVMO representative will be discovered as well. Other approaches such as ancestral sequence reconstruction[364,365] could also create thermostable BVMOs that likely show a broad substrate scope.

Lastly, the stability of the peroxyflavin should be better investigated, as uncertainties about variations in the mode of uncoupling exist.[178] While the influencing factors are largely unknown and of academic interest, improvements in oxygenation coupling will also make biotechnological BVMO reactions more reliable, efficient—and thus—realistic.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.9b03396.

Species and accession numbers associated with Figure 3 (PDF)

Table listing mutants of PAMO and the respective substrate scope (XLSX)

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Notes

The authors declare no competing financial interest.

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